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SPONTANEOUS TUMORS IN TWO COLONIES OF RATS OF THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY *

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The Wistar Institute maintains two colonies of albino rats, the Experimental Colony Strain and the Wistar Stock Colony, from which animals may be supplied to other laboratories. On occasion these have been used in research on neoplastic diseases. It seems desirable, therefore, that records of spontaneous tumors in animals of these colonies be made available.

The material to be presented was collected over a 5 year period, during which animals that developed abnormal swellings of any part of the body were submitted for study. The personnel in charge of the colonies selected these while making routine inspections of the stock.

Depending on their condition when received, the rats were either killed for immediate autopsy, or held for observation and experiment. All tumors were examined microscopically, tissues being prepared according to standard methods, and classified according to sites of origin and histological make-up. Interpretations of their actual or potential nature were based on accepted criteria, but doubtful cases will be described in some detail.

Of 468 animals selected for examination, 393 (295 females and

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98 males, or about 5 per cent of all animals dying in the group during the period) were from the Experimental Colony Strain. The remainder, 44 females and 31 males, also about 5 per cent of the animals dying in the group, were from the Wistar Stock Colony. From the entire series, 273 tumor-bearers were obtained, these being divided between the two colonies as follows: Experimental Colony Strain, 207 females and 37 males; and Wistar Stock Colony, 24 females and 5 males (see Table I).

Age Distribution and Types of Tumors

The ages of the tumor-bearing rats varied from 120 to 1020 days. The distribution of neoplasms, according to age, is shown for both colonies in Table I, cases being arranged into age groups that are separated by an arbitrary interval of 90 days.

Table I

Age Distribution at 90 Day Intervals in all Tumor-Bearing Rats

Age classes	Experimental Colony Strain		Wistar Stock Colony	
	Females	Males	Females	Males
days				
91–180	1	1	..	..
181–270	4	3	2	1
271–360	12	..	..	..
361–450	21	..	2	..
451–540	26	2	7	..
541–630	23	7	..	2
631–720	49	6	4	1
721–810	33	8	2	1
811–900	30	9	5	..
901–990	7	1	2	..
991–1080	1	..	..	..
Total tumor-bearers	207	37	24	5

In the first column of the table female rats of the Experimental Colony Strain have been listed. The actual range in age was 120–1020 days, the average age being 680 days. The age distribution followed fairly well a normal curve and irregularities probably would have been less with a larger series. Age distribution of tumor-bearing males of this colony was much less regular than for the females but the number of cases was too small for satisfactory comparison. The average age of these animals was 690 days.

The number of cases from the Wistar Stock Colony also was too

small for comparison, but the range in age corresponded fairly well with that of the 1st group, and in each group more than 75 per cent of all animals developed tumors between 451 and 900 days. The average age of females of the Wistar Stock Colony was 670 days, and of the males of this colony, 580 days.

Types of Tumors: Because of the manner in which the animals were selected for examination it was to be expected that surface tumors would predominate in the series, and this was true. However, a number of growths of internal organs were encountered also.

In Table II the neoplasms have been grouped according to their place of origin in the body and the cases have been arranged to show for both colonies, number, sex and average age of animals developing various types of neoplasms, and the histological make-up of the lesions. Indicated also are my opinions of the actual or potential growth characteristics of the tumors.

Listed in Table II are 302 neoplasms in 273 rats, 25 animals each having 2, and 2 others, 3 independent growths. For convenience of presentation, tumors of the mammary gland, although comprising the bulk of the material, have been placed last in this section.

Skin and Subcutis: Listed in this section of Table II are 22 tumors. With two exceptions, these were all of fibrous tissue origin. Tumors from females of the Experimental Colony were: 3 fibrosarcomas, 1 myxosarcoma and 1 lipoma. In males from this colony there were: 1 papilloma of the foot pad, 7 fibrosarcomas and 6 fibromas. The neoplasms from females of the Wistar Stock Colony were: 1 myxosarcoma and 1 fibroma. One male of this colony developed a carcinoma of the skin at the tip of the snout. Secondary tumors were found in the lungs in this animal. One other tumor of this group, a fibrosarcoma of the subcutis of the thorax in an Experimental Colony male, also gave rise to secondary growths in the lungs.

The average age of all animals with tumors of the skin and subcutis was 780 days, and the range in age was from 520 to 950 days. The average age of the rats that developed malignant subcutaneous neoplasms was 710 days, and again ages ranged from 520 to 950 days.

These subcutaneous tumors were located at various points on

TABLE II

Number of Tumors, Tissues Involved, Ages of Tumor-Bearers and Histological Characteristics of Tumors

Location of tumors	Experimental Colony Strain								Wistar Stock Colony							
	Cases ♀	Average age	Number of tumors E*	F**	Cases ♂	Average age	Number of tumors E	F	Cases ♀	Average age	Number of tumors E	F	Cases ♂	Average age	Number of tumors E	F
		days				*days*				*days*				*days*		
SKIN AND SUBCUTIS	5	600	..	5	14	750	1	13	2	790	..	2	1	..	1	..
Malignant tumors	4	610	..	4	7	780	..	7	1	690	..	1	1	630	1	..
GENITO-URINARY	17	720	9	8	5	430	..	..	..	..	..	..	2	430	..	..
Vagina																
Benign tumors	2	660	..	2	..	..	..	..	..	..	..	..	..	..	..	..
Malignant tumors	2	500	1	1	..	..	..	..	..	..	..	..	..	..	..	..
Uterus																
Benign tumors	3	880	..	3	..	..	..	..	..	..	..	..	..	..	..	..
Malignant tumors	7	780	5	2	..	..	..	..	..	..	..	..	..	..	..	..
Ovary																
Malignant tumors	3	500	3	..	..	..	..	..	..	..	..	..	..	..	..	..
Testes																
Malignant tumors	..	..	..	..	1	730	..	1	..	..	..	..	1	600	..	1
Kidney																
Malignant tumors	..	..	..	..	1	850	..	1	..	..	..	..	..	..	..	..
Embryonal tumors	..	..	..	..	3	200	Mixed	tumors	..	..	..	..	1	270	Mixed	tumor
DIGESTIVE TRACT																
Malignant tumors	1	420	1	..	1	900	..	1	1	240	1	..	..	..	..	..
MEDIASTINUM																
Malignant tumors	..	..	..	..	1	250	..	1	1	390	..	1	..	..	..	..
THYMUS																
Malignant tumors	1	720	1	..	1	810	1	..	1	870	..	1	..	..	..	..
OTHER LYMPHOID TISSUES																
Malignant tumors	..	..	..	..	1	780	..	1	..	..	..	..	1	690	..	1
ADRENAL	1	690	1	..	..	..	..	..	..	..	..	..	..	..	..	..
SKELETON																
Malignant tumors	2	850	..	2	3	560	..	3	1	870	..	1	..	..	..	..
MAMMARY GLAND	187	650	203	3	..	..	..	..	..	..	..	..	..	..	..	..
Single tumors	168	650	165	3	13	650	5	8	19	640	18	1	1	750	..	1
Multiple tumors	19	680	38	..	..	..	..	..	..	..	..	..	..	..	..	..
Malignant tumors	13	470	11	2	..	..	..	..	1	690	1	..	..	..	..	..
Tumors of mammary gland and of other tissues	6	780	10	4	2	540	2	2	1	870	..	2	..	..	..	..

* E = Epithelial tumors.
** F = Fibrous tissue tumors.

the body from the head to the base of the tail, no one part being distinctly favored. The fibromas presented nothing unusual in their make-up, all being small, spherical, circumscribed nodules composed of dense tissue. The fibrosarcomas varied considerably in consistence. All were locally invasive, infiltrating skin and muscles. In section they varied from richly cellular tissues with many mitoses to relatively acellular structures in which abundant collagen was formed. Two of the more cellular tumors underwent central necrosis, forming large cystic spaces filled with serosanguineous fluid. Both of these tumors were easily transplanted, being carried through 4 series of animals. Other sarcomas were not subjected to this test.

Genito-Urinary Organs: Neoplasms of various parts of the genito-urinary system developed in 24 animals.

The ages of females, all of the Experimental Colony, ranged from 360 to 940 days. The average age of those having carcinomas was 690 days and the range in age was the same as for the whole group. The average age of females with tumors of fibrous tissue equalled that of the group and ranged from 540 to 930 days.

Four of the tumors in females originated in the vagina. Two of these were fibromas, 1 occurring within the vaginal canal as a pedunculated growth, the other as a nodular mass on the serous surface. A 3rd growth also formed within the vagina as a soft body which expanded the tube and infiltrated a wide section of the wall. This was classified as a myxosarcoma. The final tumor was a squamous cell carcinoma, developing at the mucocutaneous junction of the genital tract.

Of tumors of the uterus, 2 were soft pedunculated masses of fibrous tissue attached to the endometrium, and 1 a large irregular growth of soft tissue arising from the serosa of one uterine horn and adhering lightly to the intestines. This was composed of myxomatous tissue which did not appear to be malignant. Two other tumors of fibrous tissue origin involved large segments of a uterine horn. Both were locally invasive, replacing wide areas in the wall. One was a fibrosarcoma and 1 a myxosarcoma.

Epithelial tumors of the uterus were all malignant and have been classified as adenocarcinoma, and in each case secondary growths developed within the peritoneal cavity.

The other 3 neoplasms of the genital tracts of females involved

* E = Epithelial tumors.
** F = Fibrous tissue tumors.

the ovary. These were carcinomas and all were essentially alike grossly, as well as in histological make-up. Two of these gave rise to secondary growths on peritoneal surfaces.

Tumors of the genito-urinary organs of male rats may be considered together, although both colonies were represented. Two of these growths involved the scrotal tissues of the host, 1 from each colony. Both were fibrosarcomas, which were locally destructive and extended into the peritoneal cavity as well.

Five other neoplasms involved a kidney in each host, completely replacing the organ. Four of these animals were from the Experimental Colony and, except for 1, were under 1 year of age at death. The male from the Wistar Stock Colony also was under 1 year of age. Four of the tumors were essentially similar in histological make-up, being composed of imperfectly formed epithelial structures and blood vessels which simulated nephrons and which were supported by abundant, richly cellular stroma. These growths were confined to the organ in which they developed and apparently were embryonal tumors.

The last of the renal neoplasms also replaced one kidney but, in sections, was composed of a dense mass of spindle cells which, in some areas, were arranged about an osteoid matrix. Metastases were found in the omentum. This tumor seems to have been a fibrosarcoma. The animal in which it occurred was the oldest of the group — 850 days. The average age of the other 4 was 210 days, the youngest 130 and the eldest 270 days.

Digestive Tract: Three tumors occurred in tissues of the digestive tract. One was an adamantinoma of the lower jaw in a female of the Experimental Colony. This invaded local tissues and was successfully transplanted into 2 animals. A 2nd was a fibroma of the serosa of the small intestine of a male from the same colony. The 3rd was a carcinoma of the salivary gland of a female from the Wistar Stock Colony.

Mediastinum: One male of the Experimental Colony developed a fibrosarcoma of the lower anterior mediastinal tissues and 1 female of the Wistar Stock Colony had a lymphoblastoma of this region. Extensive secondary growths were found in both animals.

Thymus: Neoplasms of this tissue developed in 3 animals. The tumors in 2 of the rats from the Experimental Colony were composed chiefly of epithelial elements of the thymic tissues, while

that of the 3rd animal was made up of small lymphoid cells. In each instance secondary growths developed in the lungs.

Other Lymphoid Tissues: Lymphosarcoma of the mesenteric lymph nodes developed in 1 male rat from each of the colonies.

Adrenal: A relatively large adenoma of cortical cells arranged as irregular cords and masses into which hemorrhages had occurred was found in a female of the Experimental Colony. This animal also had 2 other tumors.

Skeleton: Neoplasms of skeletal tissues developed in 6 animals. One of these arose at the costochondral junction, 2 involved the left femur arising near the distal end, and 1 the right femur. A 5th developed at the proximal end of the tibia, and the last involved the humerus. Only 1 of these tumors gave rise to a secondary growth, and this in the ovary. The gross characteristics and histological make-up of the growths permit their classification as osteogenic sarcoma.

The average age of animals that developed this form of neoplasm was 690 days and ages ranged from 490 to 870 days. The females were all over 800 days of age; the males were much younger, the average age being 560 days.

Mammary Glands: The mammary gland was a prolific source of tumors in animals of both strains, 77 per cent of all tumor-bearers having a growth at this site. In 207 tumor-bearing females from the Experimental Colony, this tissue produced 206 neoplasms. These developed in 187 animals, 19 of which bore 2 mammary tumors each. Of these growths, 203 were composed either of glandular and ductal epithelium, or of ductal epithelium alone, with variable amounts of fibrous tissue. Three of the growths, each occurring in separate hosts, did not contain epithelium; 2 were fibromas and 1 a lipoma.

The histological appearance of 13 tumors suggested malignancy, 11 of these being interpreted as carcinomas and 2 as sarcomas.

In 6 females of the Experimental Colony, tumors of the mammary gland were associated with growths in other organs. In 2 of these animals, 1 with 2 mammary tumors, fibromas of the uterine wall were found. The mammary tumor of the 3rd female was undergoing sarcomatous change and was accompanied by a carcinoma of the uterus. The 4th animal developed a carcinoma of the mammary gland, a fibrosarcoma of the subcutis, and a cortical

adenoma of the adrenal. In the 5th animal the accompanying tumor originated in the thymus. In the 6th there was an osteogenic sarcoma of the femur.

Thirteen males from the Experimental Colony also developed tumors of the mammary area of the subcutis. Five of these growths contained epithelium and were identical with the fibroepithelial tumors of female rats. Five others were similar to these grossly but on section proved to be fibromas. The remaining tumors were lipomas. In 2 males with fibroepithelial tumors of the mammary gland there were also malignant growths of other tissues. One had a sarcoma of the mediastinum and the other a sarcoma of the subcutis.

The females of the Wistar Stock Colony developed only single tumors of the mammary gland, all fibroepithelial except 1, a lipoma. The animal in which this last occurred also had a malignant tumor of the thymus. Histological make-up of 1 mammary tumor of this group suggested carcinoma. A lipoma was found in the mammary area of 1 male from the Wistar Stock Colony.

Thus, the great majority of tumors of the mammary glands of animals of both strains were the benign fibroepithelial growths which are known by a variety of names. For present purposes these have been called fibroadenoma.

In location these growths were distributed about equally among the right and left inguinal and axillary groups of mammae. In size, shape, consistence, color, rate of growth and histological appearance they differed widely. In size these tumors varied from multiple small nodules 1 to 2 mm. in diameter to large spheroid or discoid masses of 6 to 8 cm. Generally they were sharply outlined in the subcutis with or without the attached or adjacent mammary tissue being hyperplastic. Incision into the tumors usually exposed nodules or lobules of various size, consistence and color, more or less firmly embedded and irregularly spaced in a less dense tissue matrix. Occasional growths were uniformly firm and tough without definite subdivisions. Others were soft and spongy and often contained milk-like fluid in single or multiple cysts.

Although variation was considerable, the histological make-up of fibroadenomas from females of this series seemed to follow a basic pattern, the essential elements of which were variable amounts of ductal and acinar epithelium arranged into fairly defi-

nite lobules and distributed in stroma which also varied in amount, density and maturity. The amount of glandular tissue ranged from a few scattered hypoplastic ducts to well formed areas of apparently actively secreting acini. Usually the condition of the glandular tissue was fairly uniform throughout a tumor, but occasionally hypoplastic lobules lay adjacent to hyperplastic lobules. The extremes of variation in amounts of epithelium have been illustrated in Figures 1 and 2 which, for convenience, were taken from tumors that developed in male rats.

The microscopic appearance of fibroadenomas developing in the mammary glands of male rats did not differ essentially from that of the tumors found in females. Four of the 5 tumors contained only ductal epithelium enclosed in dense fibrous tissue. In the other tumor, both ducts and acini had formed (Figs. 1 and 2) and secretion was abundant.

The gross and microscopic features of the fibroadenomas from animals, each of which developed 2 of these tumors, differed from case to case. Often too, growths on a given animal were unequal in size and consistence, but with one exception each pair was similar histologically. Of the growths in this animal, 1 was an ordinary fibroadenoma while the other was an adenocarcinoma.

Fibroadenomas varied widely in their rate of growth. By measurements at 30 day intervals on 20 tumors, 11 of them were found to have increased from 2 to 10 times their original volume during a period of 90 days. The remainder were unchanged. As a rule tumors that were less than 2 cm. in diameter when the hosts were received grew most rapidly, but rate of growth did not seem to be correlated with the condition of the host or the histological make-up of the neoplasms. For example, 1 of the tumors that proved to be a carcinoma increased in average diameter from 1.5 to 2.7 cm., while 2 fibroadenomas grew from 2 to 4.5 cm. and 3.5 to 6.5 cm. respectively during the same period. The carcinoma developed in a rat 120 days of age.

Surgical removal of the major portion of the fibroadenomas, without disrupting the blood supply, did not cause appreciable change in 10 instances. The remnants of the tumors had not increased in size when the animals were killed 3 to 5 months after the operation.

Complete removal of the fibroadenomas from 10 rats also was

without result after 4 to 7 months, except in 1 animal. In this rat the mammary gland about the site of the original tumor underwent hyperplasia so that 30 days after the operation its outlines could be palpated. It remained in this condition for about 2 months, during which time a 2nd fibroadenoma developed in another group of mammary glands.

The malignant tumors of the mammary gland were recognized only by microscopic examination. All were taken to be fibroadenoma grossly. Secondary tumors were found only in 1 animal, and these in the lung. In the whole series of rats 12 carcinomas of the mammary gland were encountered. These seemed to be of two types. The first included 9 specimens. These have been classified as adenocarcinoma and, histologically, were characterized by dense masses of pseudoglandular tissue with relatively scanty but cellular stroma. All infiltrated adjacent structures and mammary tissue of the involved gland was hyperplastic (Fig. 3). One of this group of tumors developed in an animal that also had a fibrosarcoma of the subcutis and a cortical adenoma of the adrenal.

The second type was represented by 3 tumors from females of the Experimental Colony. In these growths the microscopic appearance suggested that a carcinoma had developed in a fibroadenoma, filling spaces formerly occupied by ducts and glands with masses or strands of cells. In 1 of these tumors glandular epithelium was undergoing squamous metaplasia (Fig. 4).

The 2 fibroadenomas in which sarcomas developed were not unusual otherwise. In both tumors portions apparently were being replaced by a richly cellular tissue composed of spindle cells arranged in irregular strands and whorls. One of the animals in which this occurred also had a carcinoma of the uterus.

Age Distribution of Mammary Tumors: The ages of the animals developing tumors of the mammary gland varied widely. Age distribution for both colonies is shown in Table III, cases being grouped into classes that increase throughout the range by an arbitrary interval of 90 days.

In the first column of this table mammary tumors in females of the Experimental Colony are arranged according to age groups. The ages ranged from 120 to 1020 days, and the frequency of tumors increased with age up to 720 days, approximately 80 per cent developing between 451 and 900 days. In the second column

of the table carcinomas of the mammary glands of females of this colony are arranged according to age. Seven of these occurred between the ages of 90 and 450 days; actually the youngest animal was 120 days of age, and 4 carcinomas developed between 631 and 810 days. In the third column mammary tumors of males of the Experimental Colony Strain are arranged according to age groups. In this series, as well as that shown in the fourth column,

TABLE III

Age Distribution at 90 Day Intervals in Rats Developing Tumors of the Mammary Gland

Age classes	Experimental Colony Strain			Wistar Stock Colony	
	All cases ♀	Carcinoma ♀	All cases ♂	All cases ♀	Carcinoma ♀
days					
91–180	1	1	..	..	..
181–270	4	2	1	1	..
271–360	11	2	..	..	..
361–450	20	2	..	1	..
451–540	21	..	..	5	..
541–630	21	..	4	2	..
631–720	44	2	5	3	1
721–810	31	2	2	2	..
811–900	27	..	1	3	..
901–990	6	..	..	2	..
991–1080	1	..	..	..	..
Total	187	11	13	19	1

mammary tumors of females of the Wistar Stock Colony, age distribution tends to parallel that in the first column which reproduces fairly well a normal curve.

Incidence of Tumors: The colonies of rats from which this material was drawn differ from the usual population groups in which incidence of neoplastic disease has been studied in that they are essentially breeding stocks. A variable fraction of their number is disposed of each year. These are of all ages.

From available records it is impossible to determine the average age of the colonies from year to year or the proportion of males to females. The census merely indicated the number of animals present at a given time.

For each year of this study the census, *i.e.* animals present at the end of the year, the numbers sold, dying and bearing tumors

during the year, and the incidence of neoplasms, both as a percentage of population and of deaths, has been given for each colony in Table IV. Colony population for each year is the sum of census, sales and deaths. Only the number of deaths and of tumor-bearers may be divided according to sex. Since all records, except number and sex of tumor-bearers, are available only by calendar years and the work was begun and ended in September,

TABLE IV

Census, Sales, Deaths, Tumor-Bearers and Incidence of Tumors as a Percentage of Population and of Deaths in Two Colonies of Rats of the Wistar Institute of Anatomy for a Period of Six Years *

Year	Census	Sales	Deaths ♀	Deaths ♂	Tumors ♀	Tumors ♂	Percentage incidence of tumors: Population	Percentage incidence of tumors: Deaths ♀	Percentage incidence of tumors: Deaths ♂
Experimental Colony Strain									
1	2579	2177	548	714	8	5	0.86	6.68	2.24
2	2789	1206	531	720	37	4	0.78	6.96	0.55
3	2316	1291	625	788	74	16	1.79	11.84	2.03
4	2124	1097	510	896	21	5	0.46	4.11	0.55
5	2479	666	682	760	28	4	0.69	4.10	0.52
6	1715	711	613	643	39	3	1.52	8.48	0.62
Wistar Stock Colony									
1	796	391	178	233	1	0	0.25	2.24	0.00
2	1040	286	240	331	9	0	0.47	3.75	0.00
3	626	74	297	421	8	1	0.64	2.69	0.23
4	290	9	204	198	5	2	0.99	2.45	1.01
5	258	50	105	147	1	1	0.36	0.95	0.67
6	178	2	81	111	0	1	0.37	0.00	0.90

* Incidence of tumors for the 1st and last years are estimated because the study was begun and ended in September. Under the column entitled "Tumors" is given the actual number of tumor-bearers.

Table IV was arranged to accommodate for this by dividing the totals according to calendar year and estimating the number of cases for the 1st and last years in terms of actual number of tumors obtained during the portion of the year covered by the study. These totals were used only to compute incidence. In the column "Tumors" is given the actual number of cases.

The census of both colonies, the sales and the tumor-bearers varied irregularly from year to year. At the same time the annual death rate also varied, from 20 to 35 per cent of the population in the Experimental Colony and from 25 to 51 per cent in the

Wistar Stock Colony. The incidence of neoplasms shows little relation to the death rate and, both as a percentage of population and of deaths, changed from year to year in the two colonies.

The proportion of tumor-bearers to total population of the Experimental Colony Strain was less than 1 per 100 during 4 of the 6 years and was never higher than 1.79 per cent. In animals of the Wistar Stock Colony the yearly incidence of tumors also was less than 1 per cent and except for 1 year was lower than in the other group.

When divided according to sex and expressed as a percentage of deaths (see last column, Table IV) the incidence of tumors was much higher in females of the Experimental Strain than in the other groups. Females of the Wistar Stock Colony were next in order of frequency, except for 1 year when no neoplasms were found, followed by males of the Experimental Strain. Males of the Wistar Stock Colony developed very few tumors but, for the last 3 years of the study, there were proportionately more cases among them than in males of the other group.

Using the estimated total number of tumor-bearers, since losses by death for the actual period of the study are not available, the incidence of neoplastic disease for all deaths in both colonies was 3.1 per cent; for all females 5.7 per cent, and for all males 1 per cent. Again using the estimated number of tumor-bearers, the ratio of neoplasms to deaths in the Experimental Colony was 6.7 per cent for females and 1.2 for males, and in the Wistar Stock Colony 2.4 per cent for females and 0.4 per cent for males.

With this material it must be remembered that animals were selected for examination by the personnel in charge of the colonies. Thus, growths of surface tissues should, as they do, predominate, and these percentages apply chiefly to external tumors.

As pointed out before, most of the surface growths (77 per cent) involved the mammary gland. The incidence of mammary tumors for all female deaths in the Experimental Colony was 5.4 per cent, and in the Wistar Colony 1.7 per cent. When taken for each of 4 yearly periods for which returns are complete, frequency of growths of the mammary gland varied from 3.9 to 11 per cent of deaths among females of the Experimental Colony, and from 0.95 to 2.5 per cent in the other group.

Malignant tumors of the mammary glands of females were

relatively uncommon. For the Experimental Colony the percentage of carcinomas was 0.31 and for fibroadenomas undergoing sarcomatous change 0.06. In the Wistar Colony 1 carcinoma occurred in 1105 female deaths.

Fibroepithelial tumors of the mammary glands of males were found only in animals of the Experimental Colony, there being 5 cases, or about 1 per 1000 deaths.

With the exception of the skin, subcutis, skeleton and genitourinary organs, all of which were the sites of relatively few neoplasms, records for other tissues probably do not represent completely their susceptibility. However, it should be noted that tumors of genital organs developed in approximately 0.6 per cent of female rats dying in the Experimental Strain, but did not occur in females of the Wistar Stock Colony.

Discussion

In considering the types of tumors found in rats of these two colonies, together with their frequency and location in the body, it must be emphasized again that all animals received for examination were selected by the personnel in charge of the colonies. Thus, in comparing these strains of rats with others, some degree of bias in favor of tumors of surface tissues must be taken into account. Perhaps very few growths developing in abdominal organs were missed because of the ease with which small abnormal masses may be palpated in this section of the body cavity of the rat. But very probably a high percentage of thoracic neoplasms was not detected.

Material drawn from each of the two colonies undoubtedly was subject equally to selection. Therefore, differences shown by the two strains in incidence of tumors generally, as well as in relative frequency of growths in the mammary glands and genital organs of females, may be of importance for those using these animals for experimental studies of neoplasms. Often rats obtained from the Wistar Institute for such investigations have not been assigned a definite colony origin in reports of the work.

On the whole, the incidence of neoplasms, expressed as percentages of deaths by sexes for the combined material, compares favorably with that given by Curtis, Bullock and Dunning [1] for a more extensive series of autopsies on rats which were derived

from several strains. But incidence of growths of the mammary gland in both sexes of the present series, as well as frequency of sarcoma of bone, was considerably higher than they reported.

Tumors of the mammary glands of rats, particularly fibroadenomas, have been studied for a considerable period.[2] As illustrated further by the present material, frequency of the spontaneous tumors varies widely in females of different strains, from less than 1 per cent up to 30 per cent or more having been reported.[3]

Prolonged subcutaneous injection of estrogenic substances,[4] as well as direct application of mixtures of ovarian and pituitary hormones to the mammary glands of rats,[5] has been found to increase the incidence of neoplasms of this tissue. And the pituitaries of male and female rats that carried spontaneous, induced or transplanted fibroadenomas have shown increased numbers of chromophobe and degranulated cells and decreased acidophilic cells.[6] Similar changes in the pituitaries, associated with irregularities of estrous cycles, have been reported in females of the Albany Strain of rats of which more than 30 per cent developed fibroadenomas.[3]

Unfortunately estrous cycles and endocrine glands were not studied in any of the animals of the present series, but in the pituitaries of females of the Experimental Colony Strain examined elsewhere abnormal cell ratios were reported in 15 of 26 animals.[7] In 2 rats chromophobe adenomas had developed. These animals were 632 days of age, about the center of the age groups in which fibroadenomas were most common in this strain, and this tumor occurred in 3 of them.

These abnormalities of the pituitary body correspond to those reported to be due to prolonged stimulation by estrogenic hormones.[8] Thus, available evidence supports the suggestion [3] that fibroadenomas and adenomas, and possibly other tumors of the mammary glands of female rats, are due to prolonged, recurrent abnormal stimulation of mammary tissue by ovarian and pituitary hormones.

Increased frequency of fibroadenoma, adenoma and carcinoma of the mammary glands of female rats (obtained from the Wistar Institute, but colony origin not stated) has been reported following forced breeding [9]; that is, breeding as frequently as possible without allowing young to be suckled. In this study, functional

activity of the mammary tissue and retained milk were stressed as factors in tumor growth.

The presence of milk within acini, ducts and cysts of fibroadenomas may be part of the response to internal secretion (pseudopregnancy). And, if it be granted that rapidly recurring pregnancies permitted more intense hormonal stimulation of mammary tissue than would have occurred normally, then the results of forced breeding are in keeping with other studies that have been discussed.

The mammary gland of the male rat normally develops an extensive system of ducts.[10] Opinions differ as to the source of the stimulus for growth of this tissue in the male, it being claimed that the testes do and also do not exert an influence, and that theelin is effective if the hypophysis be intact.[11, 12, 13]

Four of the fibroadenomas that developed in males of the Experimental Colony Strain may be regarded as fibromas in which normal mammary ducts were included as the tumors grew. Thus, these lesions would be essentially similar to simple fibrous tissue tumors of the mammary areas of males, 8 of which were found. But in the 1 remaining fibroadenoma, well developed glandular structures were formed and lactation had occurred.

If the testes have an influence in mammary development of rats, perhaps fibroadenomas of the mammary glands of males are analogous to gynecomastia in man, which develops when the testes undergo atrophy.[14] But if pituitary disorders be regarded as a factor in the development of fibroadenomas of female rats, it may be that there is a strain susceptibility to pituitary disfunction which could account for the appearances of these tumors in males, for although the mammary gland of the male rat does not respond to implantation of normal rat hypophyses,[15] it is stimulated to hyperplasia by extract of pituitaries of pregnant animals.[16] And as evidence that theelin is not a factor in the appearance of fibroadenomas of male rats, is the report that this substance does not prevent fibrosis of transplanted fibroadenoma in males or affect the rate of growth of the tumors.[17]

Summary

During a 5 year period, rats from two colonies of the Wistar Institute of Anatomy were submitted for examination whenever

spontaneous tumors were suspected, the animals being selected by the personnel in charge of the colonies.

Due probably to this means of selection a majority of the neoplasms were found to have developed in surface tissues, the mammary gland being the important focus. Much less prolific sources were other tissues of the skin and subcutis, various divisions of the genito-urinary system, and the skeleton.

Frequency of tumors in terms of deaths was approximately 3.1 per cent for all animals, and 5.7 for females and 1 per cent for males. Separated for the two colonies the percentages were 3.03 of one (6.7 per cent of females, and 1.2 per cent of males) and 1.1 (2.4 per cent of females and 0.4 per cent of males) of the other. The material has been analyzed to show age distribution and type of tumors for the colony groups.

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DESCRIPTION OF PLATE

PLATE 62

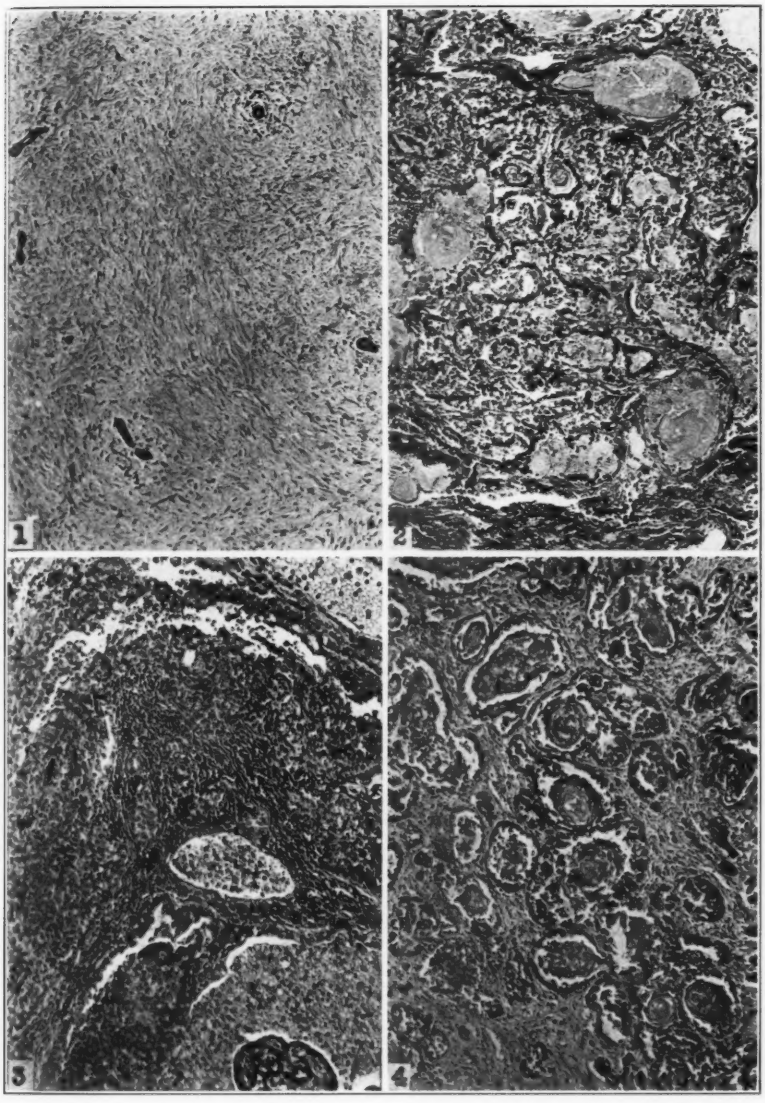
FIG. 1. Rat 855, ♂, 840 days. Fibroadenoma of mammary gland. Scattered ducts lined by cuboidal cells are enclosed in a dense stroma. × 90.

FIG. 2. Rat 749, ♂, 250 days. Fibroadenoma of mammary gland. A lobule of glandular tissue bordered by dense bands of fibrous tissue is composed of indistinctly formed acini and ducts, the latter distended with secretion. × 90.

Figures 1 and 2 represent the extremes in glandular tissue content in fibroadenomas of all animals of the series.

FIG. 3. Rat 535, ♀, 680 days. Carcinoma of mammary gland. × 90.

FIG. 4. Rat 578, ♀, 380 days. Mammary gland, the appearance of which suggests that squamous carcinoma had developed in a fibroadenoma. × 90.



Ratcliffe Spontaneous Tumors in Rats

MORPHOLOGICAL ALTERATIONS IN THE GASSERIAN GANGLION CELLS AND THEIR ASSOCIATION WITH SENESCENCE IN MAN *

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The experimental and morphological studies of Dogiel,[1, 2, 3] Nageotte,[4] Ranson,[5, 6] Cajal [7] and De Castro [8] have contributed much to our present knowledge of sensory ganglia, and for a complete review of the literature the reader is referred to these investigations.

The literature on the effect of age and the problem of distinguishing normal from senile and pathological alterations of cell cytology has remained incomplete. An earlier report by the author [9] demonstrated these alterations in sensory ganglia, and Kuntz [10] has recently described similar structural changes in aged autonomic ganglia. Although no mention of such a similarity was made, this corroborative evidence should be pointed out inasmuch as it indicates a widespread degenerative process involving both somatic sensory and visceral efferent ganglia of the body. The age factor is more directly correlated with a process of ganglion cell degeneration since there is ample clinical evidence in man which demonstrates a gradual decrease in sensitivity after the third decade (Pearson [11]). Corbin and Gardner [12] have shown that such a loss of sensitivity is due to a decrease in the number of myelinated dorsal root fibers after the third decade.

The present investigation was made in an attempt to determine whether the destructive process was of pathological etiology or merely a normal cytomorphological alteration in a neuron which accompanies human senescence. In addition, the incidence and variations of ganglion cell structure were studied to determine whether such atypical cells might serve as criteria for age determination and whether they represent neuronal expressions of degeneration, regeneration or senility.

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Material and Methods

Gasserian ganglia from individuals of different ages were used in this investigation. Only fresh ganglia in which the ante mortem clinical symptoms and subsequent postmortem findings were mild and localized were used for this study. No specimen is included that manifested generalized septicemia, toxemia, carcinomatosis or similar pathological conditions. Ganglia from both sexes were used. The males ranged from 27 months to 66 years of age, the females from 26 to 81 years.

Fixation was in ammoniated absolute alcohol, 10 per cent alcoholic chloral hydrate, or in 10 per cent neutral formalin. Following fixation the tissues were carried through the Ranson pyridine-silver and Cajal silver impregnation methods, respectively. Sections of silver preparations were cut in a craniocaudal plane at 12 μ. The formalin fixed specimens were cut at 8, 10 and 12 μ and alternate sections were stained with the Bodian protargol (1936) and cresyl violet technics.

Cell counts were made of five alternate (every fifth) sections through the center of each ganglion from patients of different ages. The various atypical cell forms and cells in the process of degeneration were tabulated. Possible error by repetitional counting was minimized, so far as possible, by placing a rectangular metal disc in the ocular to prevent overlapping fields of vision. Only cells demonstrating a distinct nucleolus were counted. The studies were made with a Leitz microscope equipped with an X15 ocular and a No. 6 objective. An oil immersion lens was used for minute details and for purposes of questionable identification.

Observations

A. Normal Ganglion Cells

The typical cells of the sensory ganglion of the fifth cranial nerve are of the unipolar type. The perikaryon possesses a smooth rounded contour and is invested in a delicate connective tissue capsule. Normal unipolar ganglion cells, as shown with low and high magnifications (Figs. 1 and 2), are readily recognized in most silver preparations. The single process arising from the cell body is occasionally observed dividing into its peripheral and

central branches. One is impressed by the frequency of subcapsular coiling of the so-called axon or stem prior to its terminal division.

Melanin pigment is present in many of the normal cells from patients of all ages. It occurs as angular granules dispersed throughout the cytoplasm, or clumped into deeply staining peripheral and perinuclear masses.

B. Atypical Ganglion Cells

While atypical cells occur most frequently in pathological sensory ganglia, they are also found in smaller numbers in the apparently normal gasserian ganglia from patients of all ages. The appearance of such cells varies extensively and no limitation can be placed on the number or complexity of the processes of a cell. In spite of this wide variation in appearance the atypical cells fall into general cell types manifesting specific characteristic features. After careful study of hundreds of diverse silver preparations, the following appears to be the logical classification of such types of cells:

1. Frayed Cells (of Cajal): This type is the most frequent atypical element observed in gasserian ganglia (see Table I). It occurs in abundance in toxic conditions and in advanced age, but is also observed in normal young ganglia. It shows a frayed and festooned outline due to the supernumerary processes arising from the soma and axon. Each process of the cell, as shown in Figure 3, terminates primarily below or within the capsule as a thickened small knob. In other instances the processes divide near the capsule into shorter branches which may or may not possess varicosities and terminal expansions. Only rarely do such processes extend beyond the limits of the capsule and in even rarer instances they may acquire a distinct capsule of thin connective tissue fibrils. Frequently numerous processes are observed arising around the entire circumference of the cell, and such cell formations give one the impression of a cog-wheel, as pointed out by Cajal.[7]

De Castro [8] has described two new cell entities which he believes to be entirely separate from the frayed cells of Cajal. Figure 4 shows an erethized cell which possesses the spiny, hooked and palm-leaf expansions proceeding from both soma and axon.

Figure 5 demonstrates the ragged (corroded) surface of a corroded cell. Formation of separate categories for these later two cell types (erethized and corroded cells of De Castro) does not seem advisable, inasmuch as they possess a pitted or excavated soma and smaller, accessory subcapsular processes in serial section. The thicker spines and processes appear to represent merely a more marked growth and hypertrophy of neurofibrils.

2. Fenestrated Cells: This type includes cells that are perforated in the region of the axon by two, three or more window-like openings. The union of such protoplasmic cordons, which bound the fenestrations, may form the definitive axon, while in other instances they may have no discernible connections with the axon. The protoplasmic loops vary from a single delicate fibril to others with short thick cordons consisting of masses of interwoven neurofibrils.

This cell type is not of common occurrence in the gasserian ganglion and in this investigation was observed only in ganglia from patients over 50 years of age. The origin of the slings and loops lies in different optical planes, making satisfactory microphotographs impossible. Therefore, no figures of this type have been presented. Their small incidence did not seem to warrant space in the table.

3. Cells Possessing End-Bulbs: The cell outgrowths that have a terminal expansion are very often seen in ganglia from patients of all ages. The terminal club shaped enlargement is composed of masses of interwoven neurofibrils and is attached to the cell of origin by either a slender or a short thick pedicle. Figure 6 shows the less frequently observed form which possesses a short, stout protoplasmic bridge between cyton and axon. Occasionally the more slender filaments leave the cell body or axon and follow a spiral course for considerable distance within the capsule before reentering the subcapsular space and presenting its terminal bulb. Similar cells belonging to this category have been described in the gasserian ganglion of the chicken, where the intrinsic structure and neurofibrils are more conspicuous (Truex [13]).

In view of the many distinct alterations in neurofibrillar size and distribution in cells with such extraneous processes it is possible that neurofibrils play an important rôle in the formation of

these outgrowths. For this reason the cells possessing end-bulbs have likewise been included with the frayed and erethized cells in Table I.

4. Cells with Nerve Arborizations: This peculiar association of nerve fibers with certain ganglion cells was considered to be quite abundant in cranial sensory ganglia by earlier investigators. In our material they have been observed in ganglia of patients ranging from 27 months to 81 years of age, but never in very large numbers. The nerve fibers entering into the intricate arborization are seldom of homocellular origin. Usually they are heterocellular nerve fibers derived from an adjacent bundle of axons which forms a plexus about the ganglion cell. Based on the nature of the plexus and its relation to the cell, the following classification has been adopted from Cajal:

(*a*) *Pericellular Plexus:* By far the most frequently observed type of arborization was that in which many fibers of uniform diameter completely encircle a ganglion cell. The fibers interlace profusely and traverse both the capsule and the subcapsular space to form a woven basket or network about the cell, as in Figure 7. The unipolar process of the cell is distinct and plays no part in the plexus formation.

(*b*) *Periglomerular Plexus:* Figure 8 clearly demonstrates the structure of this type in which the axon becomes invested with a special plexus of finer fibers which follow a spiral course. The initial coiled portion of a unipolar process has frequently been termed the glomerulus; hence the extracapsular plexus observed in Figure 8 is designated as "periglomerular." It should be pointed out that no cells of a "sympathecoid" nature were observed in relation to any of the plexus formations observed.

C. Fatty Degeneration

The degenerative process which involves the neurons of this ganglion most constantly is similar to that described by De Castro.[8] The earliest stage of this process, not demonstrable with the usual silver technics, consists of a general clumping of the cytoplasmic Nissl bodies into the large angular masses shown in Figure 9. The destruction of the neurofibrillar reticulum is initiated as a foamy area in the periphery of the cytoplasm, usually in the region of the axon hillock. Figure 10 demonstrates this

early stage and the foamy appearance is due to the formation of minute fatty vacuoles in the cytoplasm.

Further progression of the cytoplasmic degeneration with the formation of more vacuoles leads to the second stage, as shown in Figures 11 and 12. Although this stage is frequently accompanied by an increase in cell diameter, the nucleus remains normal in structure and centrally located.

The third stage results from a progressive destruction of the cytoplasm, with a fusion of the smaller vacuoles to form larger compartments, as demonstrated in Figures 13 and 14. Additional features appearing for the first time are the gradual loss in staining capacity of the axon, a marked increase in the cell diameter and the margination of the nucleus. In the later phase of this stage (Fig. 14) the degeneration invades the axon and henceforth it is not demonstrable.

The fourth or terminal stage of the fatty degenerative process is shown in Figures 15, 16 and 17. By coalescence of the individual small vacuoles larger, more discrete compartments have been formed. Subsequent fusion of the compartments leads from a multilocular to a unilocular stage, as in Figure 15. The cytoplasm and nucleus have become pushed toward the periphery and only a thin marginal layer of cytoplasm remains in contact with the indistinct plasma membrane. The remnant of the cell wall is in turn more or less blended with the surrounding capsule (Figs. 16 and 17).

Following formation of the unilocular stage, it is not uncommon to observe that the unsupported cell wall with the thin marginal layer of cytoplasm has collapsed (Fig. 16). In other instances the multilocular stage may persist until the ganglion cell has been completely destroyed. Figure 17 shows such a cell with three slender cytoplasmic girders supporting the skeleton of the cell. This degenerative process continues until the swollen ganglion cell is completely destroyed. Remaining in the region of the cytoplasm one may or may not see evidence of débris. The small pyknotic and marginated nucleus is often the last discernible cell structure.

Recent investigators attribute phagocytosis of the degenerated cell to the satellite cells. However, in view of the connective tissue reaction adjacent to the capsule I am inclined to believe

that the macrophages play a dominant rôle in the phagocytosis of such tissue wreckage. The migration of leukocytes to the area suggests also the possibility that transformed lymphocytes (histiocytes) may assist in the phagocytic process.

Many ganglion cells demonstrating fatty degeneration also bear supernumerary processes, as demonstrated in Figure 18. This consistent association indicates that neurofibrillar hypertrophy may precede or accompany fatty degeneration.

D. Proliferation of Capsular Nuclei

The connective tissue nature of the ganglion cell capsule is easily recognized by the ordinary azocarmine stain. A section of a normal capsule shows from five to eight spindle shaped nuclei which appear spherical in cross section. A wide variational increase in such capsular nuclei is observed in association with ganglion cells undergoing fatty degeneration or those demonstrating supernumerary processes. In many instances the density of the mass of nuclei is further increased by the presence of very fine nerve fibers. The fibers are of homocellular and heterocellular origin and follow complex overlapping courses among the nuclei. These features are demonstrated in Figure 19.

E. Residual Nodules

Following the destruction and phagocytosis of a ganglion cell there appears a mass of cellular and fibrous elements which literally constitutes a grave marker. The chief cellular constituents are the endocapsular nuclei interspaced with leukocytes. In addition one is able to distinguish fibroblast and satellite cells in smaller numbers with appropriate stains. Fibrous elements penetrate the cell mass of the nodule and form a maze of irregular complex skeins. These so-called "neurotizing fibers" arise as outgrowths from axons in the region of the nodule and appear to be attracted by some strong orienting agent. Such a heterogenous mass is shown in the lower field of Figure 20, although the divergent courses of the fine fibers do not allow their complete capture in this microphotograph. Also, one can see the advancing features of fatty degeneration in the cell at the top of this figure.

F. Calcification

The deposition of calcium granules occurs quite frequently in ganglia of patients over 40 years of age. It may be observed occasionally as intracellular masses, or distributed as individual granules throughout the cytoplasm. Most frequently the calcium is deposited in the capsule or intercellular spaces of the ganglion as concentric refractive corpuscles. The lamellated corpuscles or irregular bits of calcium are commonly associated with branches of the axial artery supplying the gasserian ganglion, as in Figure 21.

Additional alterations of older ganglia appear as elastic intimal thickening of the arteries and arterioles, and occasionally as complete fibrous occlusion of an arteriole. The vascular changes thus noted may play an important rôle in the degenerative processes described above.

G. Cell Counts of Ganglia of Different Ages

The results of the tabulation of cells from patients of different ages are shown in Table I. Application of statistical methods proved undesirable due to the variation of sections, inconsistency of cell distribution and the small number of specimens involved.

The percentage of cells in the fatty degenerative process, as well as the frequency of plexuses, frayed and bipolar cells in ganglia of different ages, are shown in this table. Ten individuals were examined with a total of 93,426 cells being counted and classified. Of this number, 72 per cent were considered as normal unipolar cells. They varied from 65 per cent in a female 75 years old to nearly 97 per cent in a 27 months old male child.

Cells possessing pericellular plexuses and nerve arborizations were demonstrated in each specimen but never in large numbers. They were found to be present in 1.5 per cent of the cells of a female 55 years old, whereas they were present in only 0.047 per cent of the cells of another female 56 years old. Such structures do not appear to be correlated with age or sex and comprised only 0.257 per cent of the total number of cells tabulated.

The frayed, corroded and erethized cells, and those possessing end-bulbs, were likewise variable in their distribution and constituted only 2.986 per cent of the total number of cells. They varied

TABLE I

Specimen, sex and age	Total number of cells counted	Normal unipolar cells		Pericellular plexuses		Abnormal cells (degenerated or atypical)				Bipolar cells	
						Frayed, corroded and erethized cells, cells with end-bulbs		Vacuogranular degeneration			
		No.	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%
SH 28 ♂ 27 mos.	8593	8310	96.706	9	0.104	33	0.384	234	2.723	7	0.081
SH 51 ♀ 26 yrs.	7700	6243	81.077	5	0.064	242	3.142	1206	15.662	4	0.051
SH 66 ♂ 36 yrs.	11708	7794	66.569	41	0.350	313	2.673	3558	30.389	2	0.017
SH 67 ♀ 48 yrs.	16564	10910	65.865	12	0.072	369	2.227	5273	31.834		
SH 5 ♀ 55 yrs.	7370	5173	70.189	115	1.560	464	6.295	1618	21.953		
SH 3 ♀ 56 yrs.	8415	5889	69.982	4	0.047	380	4.515	2142	25.454		
SH 61 ♂ 57 yrs.	6073	4367	71.908	3	0.049	197	3.243	1506	24.798		
SH 39 ♂ 66 yrs.	11827	8190	69.248	27	0.228	499	4.219	3111	26.304		
SH 7 ♀ 75 yrs.	6518	4265	65.434	6	0.092	54	0.828	2193	33.645		
SH 64 ♀ 81 yrs.	8658	6424	74.197	19	0.219	239	2.760	1976	22.822		
Total	93426	67565	72.319	241	0.257	2790	2.986	22817	24.422	13	0.013

from 6 per cent in a female 55 years old to 0.38 per cent in the 27 months old male child. In connection with these cells bearing abortive outgrowths and supernumerary processes it is necessary to reiterate there were no multipolar cells that could be considered as sympathetic neurons.

Fatty degeneration was the most frequently observed alteration in cell structure and was found in 24 per cent of the total number of cells counted. As one can see in the table, the distribution of this degenerative process is less variable than the above atypical cells. It was demonstrated in 2 per cent of the ganglion cells of a 27 months old child, reached its greatest frequency (33.6 per cent) in a female 75 years old, and ranged from 21.9 to 31.8 per cent in all remaining specimens between 36 and 81 years of age.

Although the number of bipolar cells observed represents only a small fraction of the total number of cells counted (0.013 per cent), it is interesting to note that they were found in diminishing numbers with age increase. They were observed in 0.081 per cent of the cells of the 27 months old male child, 0.051 per cent of the cells of a female 26 years old, and in 0.017 per cent of the cells of a male 36 years old.

Comment

In view of the data presented in Table I, it does not follow that nerve arborizations and the atypical cells can be considered as adequate criteria for age determination. The structure and distribution of the plexuses in ganglia from early childhood to old age suggest that they represent an interesting but atypical association between ganglion cells and nerve fibers. In view of our present knowledge such an arrangement cannot be rigidly classified as either degenerative or regenerative attempts of a neuron. The structural alterations of the ganglion cell which accompany the formation of frayed, erethized and corroded cells, as well as cells possessing end-bulbs, seem to place such atypical entities in the realm of degenerated neurons. Further evidence that such cells are degenerated forms may be found in their frequent association with fatty degeneration, proliferation of capsular nuclei and calcification. In addition it should be pointed out that such degenerated neurons might readily be confused with true multipolar neurons, in view of the supernumerary processes.

Indeed, Cajal [7] and other authors classify ganglion cells with multiple end-bulbs and abortive outgrowths as multipolar neurons. Strictly speaking, such cells are multipolar only in view of their possession of more than two processes without considering the nature of these processes. However, these investigators did not apply the term multipolar with the intention of denoting a motor or efferent physiological nature.

The observations recorded in Table I suggest that the clinical loss of somatic sensitivity after the third decade (Pearson [11]), as well as the actual decrease in the number of myelinated dorsal root fibers (Corbin and Gardner [12]), are the direct result of fatty degeneration in the sensory ganglion cells of the first order of neurons. The destruction of a large number of cells with their central and peripheral rami would be expected to cause a noticeable diminution in the capacity of a nerve to convey sensory impulses, as well as a loss of many myelinated fibers. Although the analogy between the percentage of cells manifesting fatty degeneration and the results of Corbin and Gardner [12] is enticing, no definite conclusions can be made from the present small number of specimens and the lack of similar data on corresponding spinal ganglion cells. However, a subsequent investigation on the spinal ganglia, now being undertaken, indicates that there is an ultimate loss of cells also in spinal ganglia. The fatty degenerative process in human spinal ganglia duplicates that described above in the gasserian ganglion. It must be pointed out, however, that ganglion cells in the early stages of fatty degeneration (Figs. 9 and 10) may retain the ability to return to complete restitution and normality. This belief is suggested by the regenerative findings of Ranson [5] in the large spinal ganglion cells following neurotomy. Ganglion cells in the terminal stages of degeneration (Figs. 12 to 17) are probably beyond ever regaining a normal healthy state.

The author is well aware that neuron vacuolation may result from delayed fixation. Ewing [14] has expressed the belief that vacuolation of ganglion cells in most instances represents postmortem changes and is devoid of pathological significance. However, vacuolation is demonstrable in fresh, formalin fixed human specimens as well as in ganglia of normal animals killed and perfused with the same fixation agent. Such observations do not appear compatible with the belief that vacuolation is primarily a

postmortem alteration of a neuron. Similarly, fatty degeneration, as observed in senile sensory ganglia, cannot be attributed solely to the ante mortem septicemia as found by De Castro [8] in vacuogranular degeneration.

Table I shows considerable variation in the number of degenerated cells after the third decade. Due to the few cases involved, the reason why fatty degeneration should occur in 30 per cent of the cells at 36, 48 and 75 years of age, and drop to consistently lower percentages (21–26 per cent) in the remaining specimens from individuals over 50 years of age, calls for no extended discussion. It may represent only individual variability which extends over a large range.

That the large number of cells demonstrating fatty degeneration may be one criterion of age, has recently received support from the tissue culture work of Weiss and Wang.[15] Transplants of thoracic spinal ganglia of 8– to 11–day chick embryos in a mixture of blood plasma and embryonic extract demonstrate somewhat correlated stages with the age of the culture transplants. The 9th day marks the end of the normal and healthy appearance of the neurons. The interesting degenerative phenomena begin in the cell with the appearance of clear vacuoles. Many of the small vacuoles become confluent and produce blisters that cause parts of the cell wall to protrude. Soon the protoplasm assumes a granular appearance and the cell walls collapse. Fixed preparations of such cells reveal the nucleus lying on the surface of the cell outside the fibrillar structure.

The above rapid life cycle of tissue culture ganglion cells suggests a recapitulation of the processes being carried on regularly in apparently normal human ganglia. The similarity of senile degenerative changes of both is found in the granular clumping, vacuole formation, margination of the nucleus, and collapsed cell walls.

While McKinniss [16] found an increase in the number of spinal ganglion cells in human fetuses between 7.5 and 13 weeks of age, no data have been presented to indicate just when such proliferation ceases. The occurrence in the semilunar ganglion of bipolar neurons in diminishing numbers until the third decade (Table I) seems to indicate that certain stages of differentiation from the bipolar to the unipolar phase may continue in man long after birth.

Summary and Conclusions

Carefully selected human gasserian ganglia obtained at autopsy from 10 individuals who were free from severe ante mortem clinical symptoms, and localized postmortem changes, were studied by means of various technics. The frequency and age distribution of normal, atypical and degenerated unipolar neurons were noted with the following conclusions:

1. Nerve arborizations and plexus formations occur in semilunar ganglia of all ages and apparently represent neither degenerative nor regenerative attempts of a neuron.

2. Atypical cells (frayed cells of Cajal, erethized and corroded cells of De Castro, and cells possessing end-bulbs) represent degenerated neurons. The dominant factor in altered cell formations appears to be neurofibrillar hypertrophy which may result in many aberrant processes on otherwise unipolar cells.

3. No multipolar cells, in the classical sense, were encountered and hence no evidence for the presence of sympathetic ganglion cells was found.

4. The atypical cells show an increase in incidence in pathological ganglia, but occur in apparently normal human ganglia of all ages as well. Such cells do not serve as a specific criterion for age.

5. Fatty degeneration of sensory ganglion cells appears to be responsible for the loss of many neurons after middle age, which may account for decreased sensitivity after the third decade in man.

6. Fatty degeneration appears to represent a senile process occurring in the normal cytomorphosis of a neuron, as well as in severe pathological conditions.

7. The observations of elastic intimal thickening in axial arteries of aged ganglia suggest that vascular disturbances may be a factor in the above degenerative processes.

8. Pigmentation in the gasserian ganglion of man is so variable that it shows very little correlation with age. Calcification is most common after 40 years of age.

9. There is evidence of postnatal differentiation of bipolar cells into unipolar cells to the third decade.

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DESCRIPTION OF PLATES

PLATE 63

FIG. 1. Normal unipolar neurons under low magnification. From a male child, 27 months old. × 450.

FIG. 2. Normal unipolar neurons with high magnification. From a female, ' 5 years old. × 950.

FIG. 3. A frayed cell of Cajal. Note the short subcapsular accessory processes. From a female, 53 years old. × 650.

FIG. 4. An erethized or irritated cell of De Castro. Note the thick, palm-leaf expansions from both the soma and the axon. From a female, 65 years old. × 650.

FIG. 5. A corroded cell of De Castro. Note the ragged (corroded) surface of the soma and the short supernumerary processes. From a male, 70 years old. × 950.

FIG. 6. A cell possessing end-bulb formation. From a male, 47 years old. × 650.

FIG. ~. Pericellular plexus about a ganglion cell. From a male, 27 months .ld. × 950.

FIG. '. Periglomerular plexus on an extracapsular portion of an axon. From male, 49 years old. × 650.

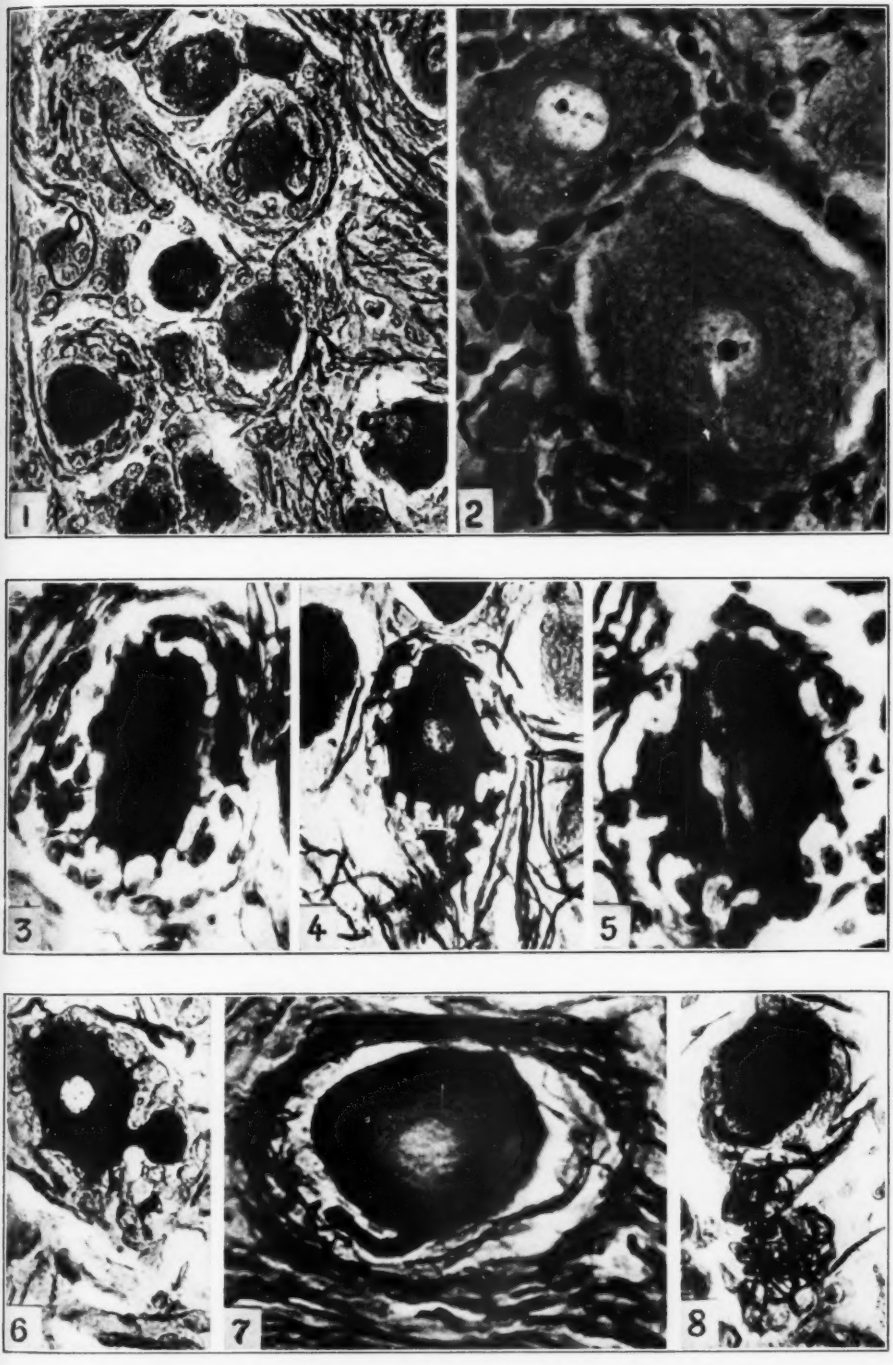
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Plate 64

Fig. 9. The earliest appearance of fatty degeneration in neurons. Note the granular clumping of Nissl bodies. From a male, 60 years old. × 760.

Fig. 10. The first appearance of minute fat vacuoles in the region of an axon hillock. From a female, 75 years old. × 1000.

Figs. 11, 12 and 13. Gradual increase in fatty vacuoles at the expense of the cytoplasm. From a female, 75 years old. × 1000.

Fig. 14. The advanced multilocular stage of fatty degeneration with margination of nucleus and loss of staining capacity of the axon. Note the increase in the cell diameter. From a female, 75 years old. × 1000.

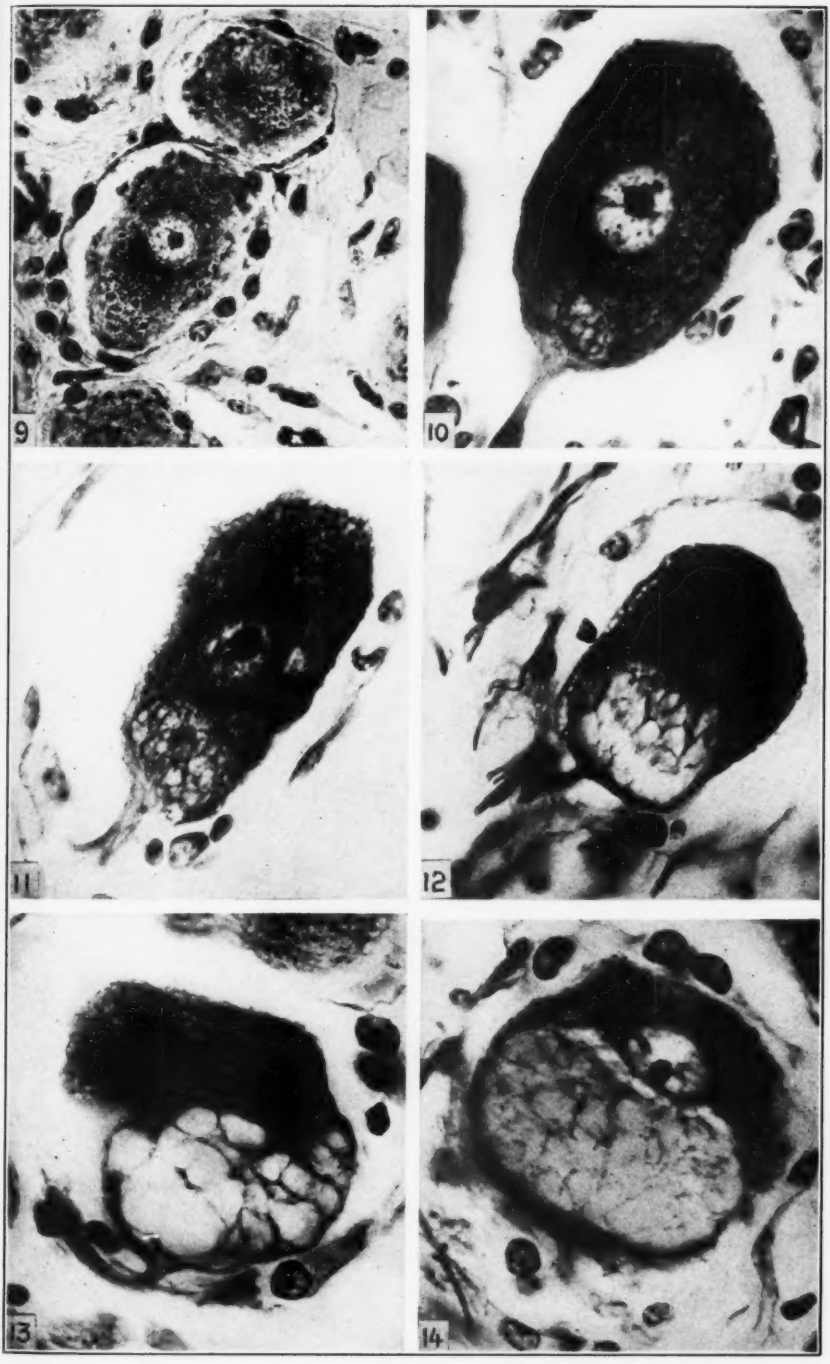
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Plate 65

Fig. 15. The unilocular stage of fatty degeneration. This results from coalescence of smaller vacuoles. From a female, 75 years old. × 1000.

Fig. 16. The terminal stage of fatty degeneration. The cell wall of the neuron has collapsed, although partially blended with the adjacent capsule. From a female, 75 years old. × 1000.

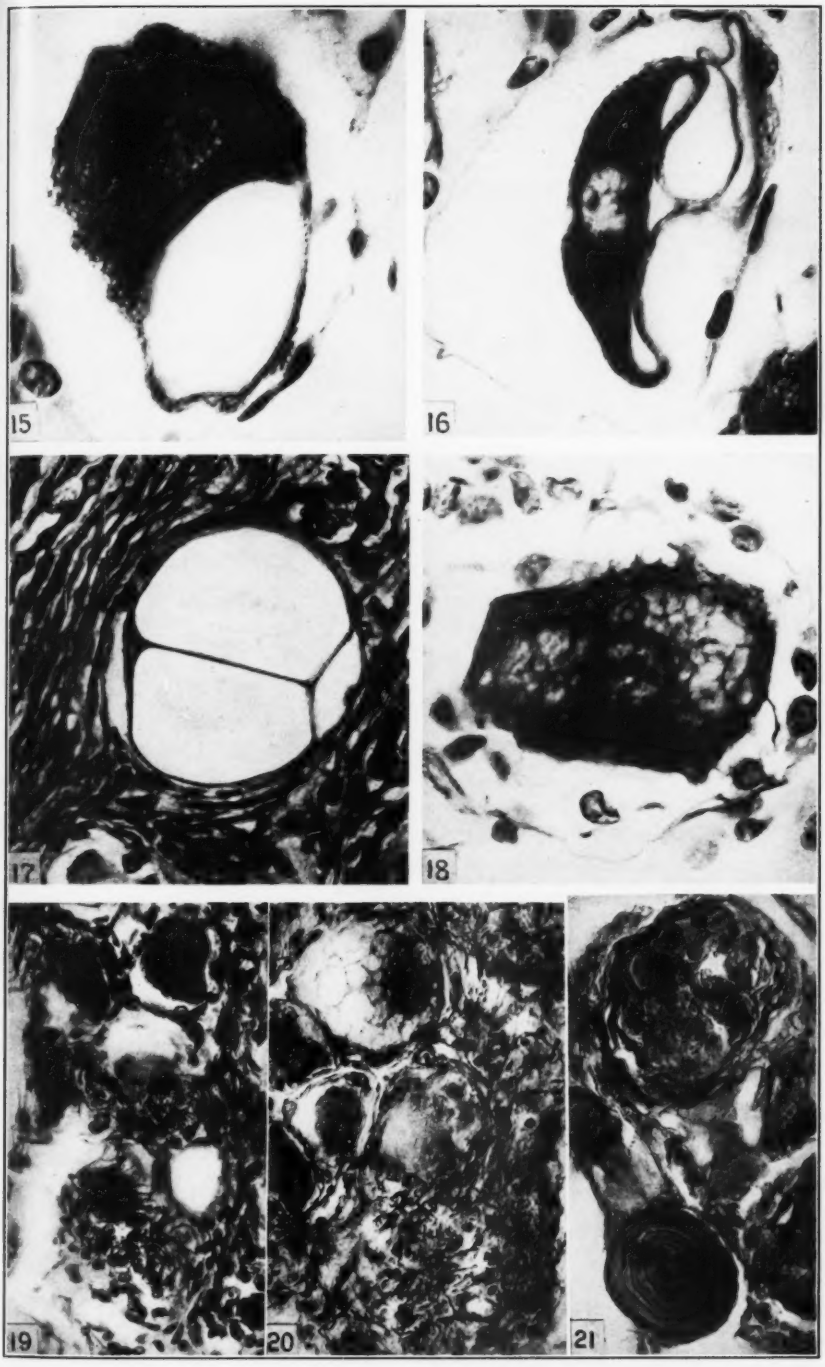
Fig. 17. The terminal stage of fatty degeneration. Multilocular conditions are retained with slender cytoplasmic girders supporting the skeleton of the cell. From a female, 75 years old. × 760.

Fig. 18. A neuron demonstrating both fatty degeneration and supernumerary processes. From a female, 75 years old. × 1000.

Fig. 19. Proliferation of the capsular nuclei. From a female, 55 years old. × 360.

Fig. 20. A residual nodule at the lower center. A neuron in the process of fatty degeneration at the top. From a female, 65 years old. × 520.

Fig. 21. A lamellated calcium corpuscle at the lower center. Note the elastic intimal thickening of the axial arterioles above. From a female, 67 years old. × 360.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ISOLATION AND CULTIVATION OF HAEMOPHILUS DUCREYI ON THE CHORIOALLANTOIS OF CHICK EMBRYOS *

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Isolation and cultivation of bacteria of the hemophilic group are tedious and somewhat uncertain procedures due to the fact that for growth on artificial mediums they require the availability of one or more specific nutrient factors. A high concentration of blood in culture mediums is the usual source of these growth factors.[1, 2] Two members of this group, *Haemophilus influenzae* and *Haemophilus pertussis*, have been cultivated on the chorioallantois of developing chick embryos by Gallavan [3] and by Gallavan and Goodpasture.[4] These previously isolated strains of bacteria were transmissible in series on chick membranes, and in the case of *H. pertussis* specific pathological lesions developed in the embryonic respiratory tract. It is the object of this report to describe the isolation and cultivation on the chick chorioallantois of *H. ducreyi* from bubonic pus from 2 clinical cases of soft chancre.† Observations on the behavior of these 2 strains in embryos have been compared with that of a previously isolated stock strain.§

EXPERIMENTAL PROCEDURE

Isolation: The technic of exposing the chorioallantois of chick embryos has been described by Goodpasture and Buddingh.[5] Membranes of 11-day old embryos were inoculated with 2 to 3 drops of pus aspirated aseptically from the lymph nodes in 2 clinical cases of soft chancre. Openings in the shells were ringed with a vaseline-paraffin mixture and closed with a sterile coverglass. Eggs were subsequently incubated at 37° C. Smears made after 24 hours incubation did not always show bacterial growth. Positive smears were found at 48 hours in each case. Seven of 10

* Aided by a grant from the John and Mary R. Markle Foundation.

† Case 1 from the Nashville General Hospital, through the courtesy of Dr. W. A. DeMonbreun. Case 2 from the Vanderbilt University Hospital, Nashville, Tenn.

§ The Ellison strain was kindly supplied by Dr. E. S. Sanderson of Augusta, Ga.

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membranes inoculated with material from Case 1 and examined at 72 hours showed a fairly abundant growth of a small, faintly staining, Gram-negative streptobacillus. All membranal cultures were pure except 1. Five of the 7 embryos were dead. Growth on dead embryos was more abundant than on the living ones. Bacilli were present in amniotic and allantoic fluids as well as on the membranes. They occurred in tangled chains of 30 to 40 units and their frequent arrangement in so-called "skeins" was characteristic.

Gross membranal lesions were exudative and progressive through 72 hours, at which time all membranes inoculated with pus had necrotic areas up to 2.5 cm. in diameter. There was often complete perforation of the membrane. Cells of less extensively involved membranes proliferated at the edges of the necrosis and the lesions healed with regression of the infection in from 5 to 8 days.

Subcultures made to whole rabbit blood, clotted and heated at 56° C. for 15 minutes, were uniformly positive. Very small, glistening, almost transparent colonies developed on 10 per cent blood agar plates inoculated with material from infected membranes. Infusion broth and slants were negative. Growth characteristics on blood clots were typical as compared with cultures of a known strain.

In this series recovery of the bacillus from only 2 clinical cases was attempted; each attempt was successful both on embryos and on artificial mediums. Isolation of the strain from Case 2 was entirely comparable with that from Case 1 described above. It is interesting to note, however, that material from Case 1 stood 24 hours in a refrigerator before being inoculated, and also that the bacillus grew neither on blood nor on membranes incubated at 39° C., although meningococci were not apparently inhibited when incubated at the same time on both artificial mediums and embryos.

Cultivation: Further cultivation of these 2 strains of Ducrey's bacillus on chick embryos was compared with a stock strain.

(*a*) *Membranal Culture:* At 48 hours transfers were made from the originally infected membranes to other 11-day old membranes by wire loop. Mild exudative lesions developed on these 2nd generation membranes with slight growth of the microorganism

up to 48 hours. The exudate consisted of a number of polymorphonuclear leukocytes but the active phagocytes were mononuclear cells. After 48 hours the bacilli gradually disappeared from smears and the lesions became dry. Transfers by loop on the 3rd and 4th days failed to induce appreciable 3rd generation lesions.

Inoculation of membranes with either of the 3 strains from cultures on blood induced fairly characteristic gross lesions. No significant difference in the behavior of the 3 strains on membranes was observed. With growth of the bacilli there was an immediate, mildly exudative membranal reaction. Bacilli continued to grow and the exudate increased through 48 to 72 hours. Infected membranes usually ulcerated, and after 96 hours contracted around the ulcer; the exudate became dry and the bacilli became increasingly more difficult to demonstrate in smears. After inoculation from blood culture or after serial passage, no extensive necrotic lesions developed such as appeared after inoculation with pus.

Each strain was transmitted through 5 serial passages with difficulty. The use of 10-day or 11-day old embryos was more favorable to maintenance of the cultures than older ones. Heavy inoculations from one membrane to another at 48 hours was necessary. Slight growth of the organisms after each transfer was evident but each serial lesion appeared to have fewer bacilli at the 48 hour interval. During 5 serial passages neither strain became adapted to rapid growth on membranes. The lethal effect of infection for embryos was negligible except where pus was used as the inoculum.

(*b*) *Amniotic Culture:* Since the heaviest growth of bacilli occurred in the amniotic fluid of dead embryos, an attempt was made to culture the bacilli serially in amniotic fluid of living embryos. The amnion was caught with sterile forceps and pulled up through a small slit in the chorioallantois. Inoculations in 0.05 to 0.1 cc. amounts of serum from blood cultures were made into amniotic fluid with a needle and syringe. The amnion was released and allowed to fall back into normal position. A few bacilli were consistently found in smears from the fluid and could be recultured on blood even after 4 to 5 days. Transfers by capillary pipette to amniotic fluid of other embryos established a 2nd generation but further serial transfer was inconsistent. Here again the indication was that the bacilli in the fluid grew feebly after each transfer.

Diluted and undiluted allantoic and amniotic fluids were used as culture mediums *in vitro*. A fairly abundant growth took place in these fluids but they are not preferable mediums.

Microscopic Examination

At 24 hour intervals membranes and living embryos were fixed in Zenker's fluid plus 5 per cent acetic acid. Cross sections of whole embryos were cut at different levels of the body for histological study. Sections were stained with hematoxylin-eosin and with iron hematoxylin-fuchsin-methyl green for bacteria.[6] Infections with the 3 described strains of Ducrey's bacillus indicated no differences in pathogenicity. The strain from Case 2 appeared in sections to have grown most readily.

(*a*) *Infection of the Chorioallantois:* Microscopic sections through chorioallantoic membranes inoculated with blood cultures and fixed at 24, 48 and 72 hours show the bacilli growing in tangled masses. A mild inflammatory reaction is present. There is ulceration of the ectoderm and mesoderm with considerable necrosis and liquefaction in the center of the lesions. The bacilli appear in a succession of colony-like concentrations at the base and periphery of the ulcers closely associated with the intact tissue. Mononuclear cells of the exudate are actively phagocytic. Extension of the lesion is vertical into the mesoderm rather than lateral. The inflammatory reaction increases up to 72 hours followed by proliferation of ectoderm and mesoderm around the area of ulceration. The continuity of ectoderm may be reestablished. As healing proceeds, the bacilli seem to be pushed to the surface into a dry, unfavorable environment from which they gradually disappear.

Membranal lesions of the second and subsequent generations are decidedly milder and heal more rapidly. No metastatic lesions were found in the bodies of embryos.

(*b*) *Infection of the Amnion:* Following inoculation into the amniotic sac Ducrey's bacillus induces in the amnion lesions comparable to those in the membrane. The bacilli appear to localize on the amnion and to grow into it in the same colon-like masses. There is less pronounced ulceration and no noticeable liquefaction. A few cases exhibit a marked infiltration with polymorphonuclear leukocytes. One case shows the bacilli eroding

the amniotic blood vessels but no metastatic lesions are found. No respiratory infections are evident. A mild peritonitis was induced in 1 case by extension of the bacilli into the abdominal cavity from the extraembryonic coelom. Small clumps of bacilli are present in the abdomen and the cells of the peritoneal exudate have phagocytosed a great many of them.

Discussion

Haemophilus ducreyi, as shown by these experiments, consistently induces characteristic 1st generation lesions in the extraembryonic membranes of chicks. The development of these pathological lesions is associated with growth of the bacilli in easily detectable numbers. These facts are of some significance because there is no experimental animal, with the possible exception of the monkey,[7] in which various investigators have been able consistently to induce infection with Ducrey's bacillus. Frei's [8] review of the experimental work on rabbits, dogs, guinea pigs, mice and cats indicates that these animals are not suitable either for diagnostic or for experimental work with this bacillus. More recently Maximowa [7] described regularly positive infections in rabbits both with pure cultures and with material from human patients. Until some readily available animal is found in which specific repeatable lesions can be induced, experimental investigation of infection with Ducrey's bacillus will be uncertain. In our experience with the methods described the bacillus was easily isolated from clinical material; characteristic 1st generation membranal lesions were repeatedly induced by inoculations with pure cultures; and in each instance the bacilli were easily demonstrable in smears, in microscopic sections, and by subculture on appropriate artificial mediums. Since such pathological lesions can be experimentally induced, problems in immunity and therapy may be approached. For example, observations on the behavior of the bacilli on the chorioallantois or in the amnion in the presence of intravenously injected antiserums or drugs such as sulfanilamide are possible.

However, this bacillus seems to be of comparatively low pathogenicity for chick embryos. Without concurrent injury to the membrane the bacilli are unable to invade it or to grow well on its surface. Injury to the membrane caused by pus or by rabbit blood cells introduced with blood cultures establishes a portal of

entry and an environment favorable for their growth. Tangled masses of bacteria develop in a zone of injured membrane between the dead and the apparently healthy tissue. These bacteria stimulate an inflammatory reaction; phagocytic mononuclear cells are active. There is little tendency for the microorganisms to invade the deeper portion of the membrane and no metastatic lesions develop.

The membranal lesion is distinctive when compared with those of other bacteria.[9] Serial membranal passage is difficult. The primary reason for this is probably the low pathogenicity of the bacillus for chick embryos. On the other hand, since the bacilli grow in the tissues in a tight colony-like arrangement, inoculations from one membrane to another may consist of relatively few infective units as compared to more easily dispersed bacterial cultures. Thus, a physiological factor and a mechanical difficulty may combine to render uncertain the development of membranal lesions in series.

Summary

1. *H. ducreyi* induces characteristic 1st generation lesions in the chorioallantois and amnion of chick embryos. The development of these lesions is associated with demonstrable growth of the bacilli.

2. This bacillus has been isolated on the chorioallantois of chick embryos from 2 clinical cases of soft chancre.

3. Cultivation of these 2 strains on the chorioallantois and on the amnion of chick embryos has been compared both grossly and microscopically with a stock strain.

4. The applicability of the methods described for further experimental work is considered.

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DESCRIPTION OF PLATE

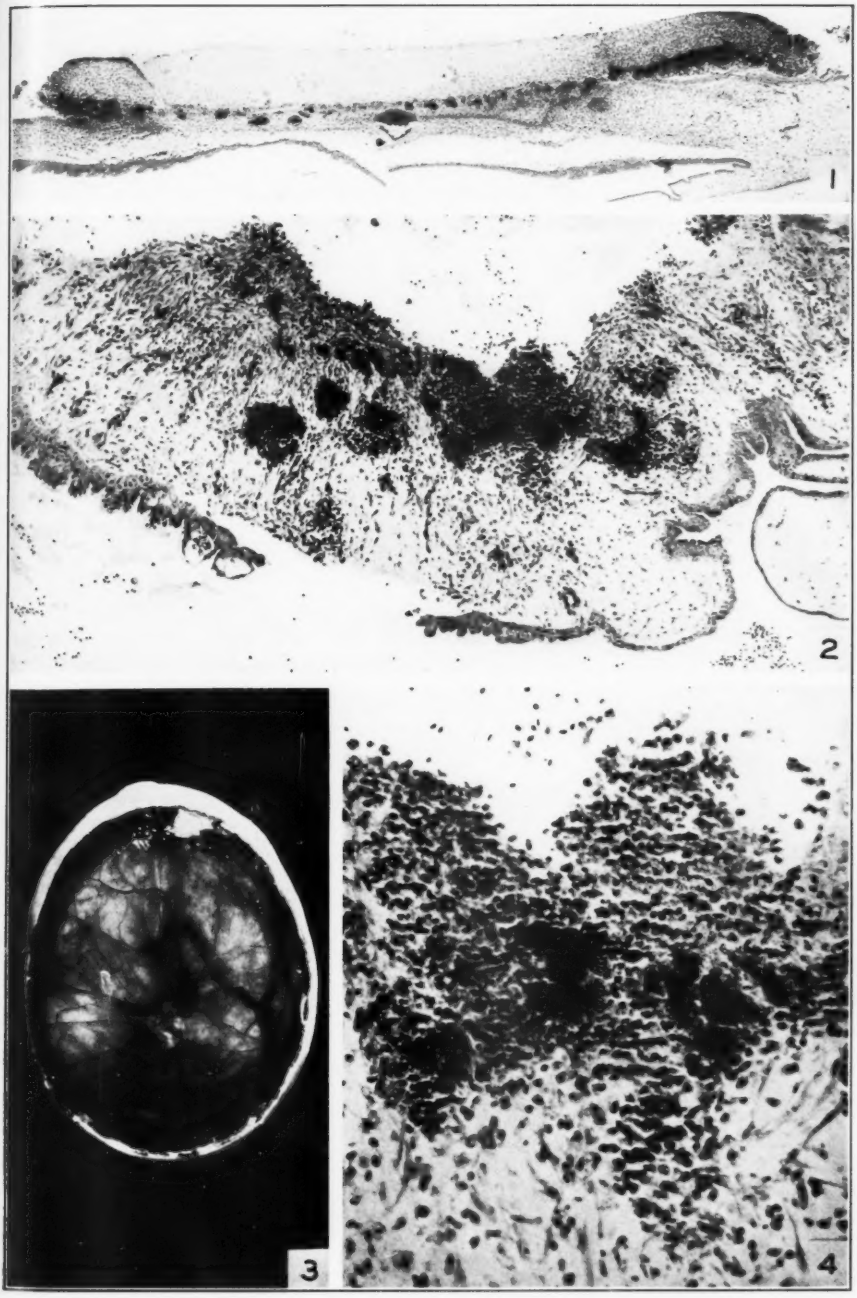
PLATE 66

FIG. 1. Section of an ulcerative chorioallantois showing a 72 hour lesion due to inoculation with *H. ducreyi* (dark masses at the base of the ulcer). There is a mild inflammatory reaction. Strain 2. × 34.

FIG. 2. Section showing a localized infection of the amnion at 72 hours (subamniotic injection). The bacilli occur in colony-like masses associated with an inflammatory exudate. Strain 2. × 75.

FIG. 3. Gross lesion on the chorioallantois at 72 hours after inoculation from a blood culture of Ducrey's bacillus. Stock strain. The membrane has ulcerated but the lesion is well localized.

FIG. 4. Higher magnification of Figure 2 showing colonies of bacilli surrounded by a leukocytic exudate composed largely of mononuclear phagocytes. × 250.



Anderson and Snow Cultivation of Haemophilus Ducreyi

PHYSIOLOGICAL HYPERTROPHY OF THE PARATHYROIDS, ITS CAUSE AND ITS RELATION TO RICKETS *

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Physiological hypertrophy of the parathyroid glands has been described in rickets by Erdheim,[1] Ritter,[2] and Pappenheimer and Minor,[3] and in renal disease by Bergstrand,[4] Hubbard and Wentworth,[5] Pappenheimer and Wilens,[6] and Shelling and Remsen.[7] Parathyroid hypertrophy has also been produced experimentally with low calcium diets by Marine,[8] and Luce,[9] and with phosphate injections by Drake, Albright and Castleman.[10] These last mentioned workers have established by experimental procedures that the opinion expressed by several former investigators was correct, namely, that hyperphosphatemia causes parathyroid hypertrophy. They state, however, that "it will require further studies to show whether hyperphosphatemia causes the hyperplasia directly or indirectly by producing a hypocalcemia." Pierre, de Boissezon and Lombard [11] have recently confined the findings of Drake, Albright and Castleman, and have found further that injections of calcium gluconate cause hypoplasia of the parathyroids of animals.

To settle whether hypocalcemia or hyperphosphatemia is the factor that directly controls parathyroid hypertrophy is by no means a simple matter. The difficulty lies in the reciprocal relationship which exists between the calcium and the phosphorus of the blood. This usually acts to effect a decrease in the concentration of one element if the concentration of the other is increased, and *vice versa*. By certain dietary procedures we have, however, attained a measure of success in obtaining each of these conditions without the other. Furthermore, we have performed experiments to clarify the relationship commonly assumed to exist between rickets and parathyroid hypertrophy. It is easy to visualize parathyroid hypertrophy in low calcium-high phosphorus rickets. But

* Received for publication November 1, 1939.

rickets can be produced with equal ease by a high calcium-low phosphorus diet, and in this type of rickets there is neither hyperphosphatemia nor hypocalcemia. We thought it would be interesting to study the parathyroids in both types of rickets to find whether they always enlarge in rickets, or only when rickets is produced by conditions leading to a hypocalcemia.

Material and Methods

Rats were used as experimental animals. They are admirably adapted to this type of work as each rat has only two parathyroid glands and these are embedded, one on each side, in the corresponding lobe of the thyroid gland. In only 1 of over 30 animals whose thyroid glands were serially sectioned was a parathyroid gland missing from its site in thyroid tissue. This animal was discarded. In none of the animals was there more than one parathyroid gland in each lobe of the thyroid gland.

The diets used are described with each separate experiment.

The method we employed to obtain figures relating to parathyroid volume requires explanation.

As the parathyroid glands of the rat are extremely small and embedded in thyroid tissue, they cannot be directly measured or weighed with any degree of accuracy. Hence, we employed an indirect method. Each thyroid gland was dissected out, fixed in Bouin's solution, embedded in paraffin and sectioned serially. The sections were stained with hematoxylin and eosin.

We were not concerned in this study with the attempt to determine the volumes of the various parathyroid glands in any standard units such as cubic millimeters. We merely desired figures that would compare with one another in the same ratios as would actual volumes. Our figures relating to parathyroid volume are therefore expressed in squares of graph paper, the number of squares being those located under a curve which was plotted as follows:

Sections of thyroids were examined consecutively until parathyroid tissue appeared. From this point on the sections were numbered consecutively. Each tenth section was examined and the greatest length and width of the oval parathyroid tissue present was measured with an eyepiece micrometer. Length multiplied by breadth gave a figure that was not the area of the parathyroid

tissue in the section but one proportional to the area. A curve was then plotted between the length-times-breadth figures and the serial numbers of the sections. The number of squares of graph paper under the curve so obtained is proportional to the volume of the gland, and hence satisfactory for our purpose. It should be pointed out that within any single experiment the graphs were all made on the same scale, but that the same scale was not used for all experiments. Hence figures of parathyroid size given for one experiment are not comparable with figures given in another, *i.e.* figures relating to parathyroid volume are only comparable within a single experiment.

Experiment 1

This experiment was performed to see if the parathyroids hypertrophy in low phosphorus rickets.

Ten young rats of approximately the same size and weight were selected and divided into two equal groups. The first group was fed a Steenbock diet with 3 per cent calcium carbonate plus 5 per cent yeast.* The second group was fed a normal diet. After 3 weeks the animals were anesthetized and the pooled blood from each group was taken for calcium and phosphorus estimations. The rats were then killed and their skeletons X-rayed. Knee joints were dissected out and fixed and decalcified in Bouin's solution, after which paraffin sections were prepared. The size of the parathyroid glands in these rats was determined by the method previously outlined.

The results of this experiment are recorded in Table I.

* The Steenbock diet used in these experiments consisted of

Ground yellow corn	76%
Gluten flour	20%
Calcium carbonate	3%
Sodium chloride	1%

Dried brewer's yeast (5 per cent) was added to this diet to aid growth. To obtain definite rickets the animal must grow during its regimen on the deficient diet.

It should be noted that a diet deficient only in vitamin D does not produce rickets in the rat. To produce rickets the diet must be unbalanced in other respects. The above diet, for instance, contains sufficient calcium carbonate to interfere with phosphorus absorption. Without the calcium carbonate, or with greatly reduced calcium carbonate, the diet is deficient in calcium. Thus, by varying the amounts of calcium carbonate either low phosphorus or low calcium rickets can be produced.

The normal serum phosphorus of the rat is higher than it is in man. The normal figure is higher in young rats than in older ones.

TABLE I

Effect on the Parathyroid Glands, Blood and Bones of Feeding Young Rats for 3 Weeks (1) a Diet Designed to Produce Low Phosphorus Rickets, and (2) a Normal Diet

	Volume * of two parathyroid glands			Average weight of rats in group	Serum calcium	Serum phosphorus	Histological examination of bone
	Smallest volume in any rat	Largest volume in any rat	Average volume in group				
				gm.	*mg./100 cc.*	*mg./100 cc.*	
Group 1. Rats fed a diet to produce low phosphorus rickets	83	182	121	101	9.4	2.3	Severe rickets
Group 2. Rats fed a normal diet	146	209	168	135	10.2	8.5	Normal

* Volume expressed in squares of graph paper (see Material and Methods).

Conclusions from Experiment 1

The parathyroid glands of the rachitic animals were smaller than those of the controls, but the relationship between size of the parathyroid and body weight was approximately the same in both groups. There was no evidence in this experiment to show that low phosphorus rickets is associated with parathyroid hypertrophy.

Experiment 2

This experiment was performed to find out if parathyroid hypertrophy developed in rats with low calcium rickets. It was controlled by a group of animals with low phosphorus rickets.

Ten young rats of approximately the same age and size were selected from the colony and were divided into two groups of 5 each. Both groups were fed a Steenbock rickets-producing diet plus 5 per cent yeast. Three per cent calcium carbonate was included in the diet of the first group, but only 0.5 per cent calcium carbonate was included in that of the second group (see footnote). At the end of 3 weeks the rats in this experiment were treated similar to those of Experiment 1.

The results of this experiment are recorded in Table II.

Conclusions from Experiment 2

Rats with low calcium rickets demonstrated marked hypertrophy of the parathyroid glands, in comparison with rats with low phosphorus rickets. As we have shown in Experiment 1 that low phosphorus rickets does not affect the size of the parathyroid glands, and as the blood phosphorus level in the animals with hypocalcemia was within normal limits, we can conclude that hypocalcemia is the cause of the parathyroid enlargement.

Experiment 3

This experiment was designed to confirm that hypocalcemia is the cause of parathyroid enlargement, and that it is effective in animals somewhat older than those used in rickets experiments.

Twelve rats, 4 weeks old, were divided into two groups of 6 each. One group was fed an adequate diet, the other group a mineral deficient diet (Robertson [12]). The animals were fed these diets for about 6 weeks, when they were killed. The blood from

TABLE II

Effect on the Parathyroid Glands, Blood and Bones of Feeding Young Rats for 3 Weeks Diets Designed to Produce (1) Low Phosphorus Rickets, and (2) Low Calcium Rickets

	Volume * of two parathyroid glands			Average weight of rats in group	Serum calcium	Serum phosphorus	Histological examination of bone	X-ray observations on bone
	Smallest volume in any rat	Largest volume in any rat	Average volume in group					
				gm.	*mg./100 cc.*	*mg./100 cc.*		
Group 1. Rats fed a diet to produce low phosphorus rickets	78	98	89	85	10.1	2.0	Severe rickets	Severe rickets
Group 2. Rats fed a diet to produce low calcium rickets	191	297	258	70	3.9	8.0	Severe rickets	Severe rickets

* Volume expressed in squares of graph paper (see Material and Methods).

TABLE III

Effect on the Parathyroid Glands and Blood of Feeding Rats 4 Weeks Old Mineral Deficient and Normal Diets for 6 Weeks

	Volume * of two parathyroid glands			Average serum calcium of large group of similar rats	Average serum phosphorus of large group of similar rats
	Smallest volume in any rat	Largest volume in any rat	Average volume in group		
				mg./100 cc.	*mg./100 cc.*
Group 1. Rats fed a mineral deficient diet	253	468	318	7.3	8.5
Group 2. Rats fed an adequate diet	205	306	243	10.2	7.7

* Volume expressed in squares of graph paper (see Material and Methods).

these animals unfortunately met with an accident so that accurate calcium and phosphorus readings could not be made. But as this adequate diet and this particular mineral deficient diet was being used in a great number of similar animals in the course of an extensive experiment, we were able to obtain calcium and phosphorus estimations from a large number of animals from both groups.

The results obtained in this experiment are recorded in Table III.

Conclusions from Experiment 3

We did not obtain the weights of the rats whose parathyroids were sectioned in this experiment. But in a large group of rats being fed similar diets it was apparent that the animals that received an adequate diet were more than half as large again as those that received the mineral deficient diet for the length of time noted in our experiment. Thus, the enlargement of the parathyroid glands in relation to body weight was even greater in the mineral deficient animals than appears by directly comparing the volume of their parathyroid glands with those of the control animals. This experiment, we think, shows that parathyroid enlargement is caused by hypocalcemia because the blood phosphorus in the mineral deficient animals was within normal limits. But even so, it was slightly higher than the blood phosphorus level of the control animals; so, to exclude the possibility that this slight elevation of phosphorus caused the parathyroid enlargement, we planned another experiment.

Experiment 4

This experiment was designed to determine whether a hyperphosphatemia in the absence of a hypocalcemia would cause parathyroid hypertrophy.

Ten young rats of approximately the same weight and size were selected and divided into two groups of 5 each. The first group was fed a normal diet, and the second group was fed a normal diet plus 3.5 parts of tricalcium phosphate and 2.5 per cent phosphoric acid. At the end of 3 weeks the rats of both groups were killed, the pooled blood from each group was tested for calcium and phosphorus, and the size of the parathyroid glands of 3 animals of the

TABLE IV

Effect on the Parathyroid Glands and Blood of Feeding Young Rats for 3 Weeks (1) a Diet Designed to Raise the Blood Phosphorus without Lowering the Calcium, and (2) a Normal Diet

	Volume * of two parathyroid glands			Average weight of rats in group	Serum calcium	Serum phosphorus
	Smallest volume in any rat	Largest volume in any rat	Average volume in group			
				gm.	*mg./100 cc.*	*mg./100 cc.*
Group 1. Rats fed a normal diet	214	307	269	150	11.6	9.7
Group 2. Rats fed extra calcium phosphate and phosphoric acid	209	294	265	147	11.3	11.7

* Volume expressed in squares of graph paper (see Material and Methods).

first group and of 4 of the second group was estimated by the method previously described.

The results of this experiment are recorded in Table IV.

Conclusions from Experiment 4

Hyperphosphatemia in the absence of hypocalcemia does not cause physiological hypertrophy of the parathyroid gland.

Comment

Relationship of Parathyroid Enlargement to Rickets: Experiment 1 showed that parathyroid hypertrophy does not occur in low phosphorus rickets. Experiment 2 showed that extreme parathyroid hypertrophy develops in low calcium rickets. Hence, we conclude that parathyroid hypertrophy is not necessarily related to rickets but occurs in that condition only when there is a deficiency of calcium in the blood.

Hypocalcemia as the Cause of Parathyroid Hypertrophy: It was pointed out in our introduction that there is no clear evidence as to whether parathyroid hypertrophy is instituted by a low blood calcium or by a high blood phosphorus. In the absence of proof to the contrary, it would be hazardous to assume both conditions were not present in all the studies that have been reported. In two of our experiments (2 and 3) we showed that a low blood calcium caused parathyroid hypertrophy in the absence of hyperphosphatemia, and in our last experiment (4) we showed that hyperphosphatemia in the absence of a hypocalcemia did not cause parathyroid hypertrophy. Therefore, we conclude that hypocalcemia and not hyperphosphatemia is the primary cause of physiological hypertrophy of the parathyroid gland.

Summary and Conclusions

1. Parathyroid hypertrophy is not necessarily associated with rickets; it occurs only in low calcium rickets, and not in low phosphorus rickets.

2. Parathyroid hypertrophy develops when the blood calcium level is low and the phosphorus at normal levels.

3. Parathyroid hypertrophy does not occur when the blood phosphorus is raised and the blood calcium is kept at normal levels.

4. Hypocalcemia, instead of hyperphosphatemia, is the primary cause of physiological hypertrophy of the parathyroid glands.

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STUDIES IN CARCINOGENESIS *

IX. Hydrocarbon-Cholesterol Pellets in Strain D Mice

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Experiments with hydrocarbon-cholesterol pellets, made according to the method of Shear,[1] have been reported by Ilfeld,[2] Wolbach,[3, 4] Andervont,[5, 6] Peers,[7] and Shear and Lorenz.[8] For comparison with the earlier investigation by Shear and Lorenz in which cholesterol pellets containing from 0.001 per cent to 5 per cent 1:2:5:6-dibenzanthracene were implanted subcutaneously in albino mice, cholesterol pellets containing these same concentrations of 1:2:5:6-dibenzanthracene, of 3:4-benzpyrene, and of 20-methylcholanthrene, as well as control cholesterol pellets containing no carcinogen, were implanted in strain D mice.†

The preparation of the pellets and the experimental procedures were similar to those employed in the previous experiments. The strain D mice used were young animals of both sexes. In each experiment the length of the pellets was varied, usually between 3 mm. and 25 mm.; the weight of the corresponding pellets ranged from about 7 mg. to 60 mg. After the introduction (5/13/35–6/14/35) of the carcinogen-containing pellets subcutaneously in the interscapular region, the mice were not subjected to any further experimental treatment.

The experiment was terminated after 11 months because the mice were in poor condition.

Dibenzanthracene-Cholesterol Pellets

Cholesterol pellets containing 5, 1, 0.1, 0.01 and 0.001 per cent 1:2:5:6-dibenzanthracene were implanted in 20, 20, 10, 10 and 10 mice, respectively.

* Received for publication December 1, 1939.

† Obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

In the group that had received 5 per cent pellets sarcomas developed in 3 mice between the 6th and 8th months. One sarcoma was obtained in the group that had received 1 per cent pellets. No sarcomas developed in the mice that had received pellets containing 0.1 per cent or less of this hydrocarbon (see Table I).

TABLE I

Tumor Production by Hydrocarbon-Cholesterol Pellets in Strain D Mice

Hydrocarbon content	Number of mice			Total number of tumors in 11 mos.
	Initial	6 mos.	11 mos.	
1:2:5:6-Dibenzanthracene				
%				
5	20	15	7	3
1	20	13	3	1
0.1	10	7	3	0
0.01	10	7	5	0
0.001	10	10	4	0
3:4-Benzpyrene				
%				
5	10	4	1	4
1	20	11	3	0
0.1	10	9	2	0
0.01	10	10	5	0
0.001	10	9	8	0
20-Methylcholanthrene				
%				
1	10	7	3	4
0.1	10	9	5	0
0.01	10	10	6	0
0.001	10	10	4	H*
Controls (no hydrocarbon)	30	29	13	0

* Hemangiomatous lesion in 1 mouse and hemangiomas in 2 others. (See page 289.)

BENZPYRENE-CHOLESTEROL PELLETS

Cholesterol pellets containing 3:4-benzpyrene, in the same five concentrations as in the preceding experiment with dibenzanthracene, were implanted in 5 groups of mice; there were 10 animals in each group except that 20 mice were employed for the 1 per cent pellets.

The 5 per cent pellets produced tumors in 4 mice between the 4th and 11th months. No sarcomas were obtained in the mice that had received pellets containing 1 per cent or less of this hydrocarbon.

Methylcholanthrene-Cholesterol Pellets

It was surmised that pellets containing 5 per cent of 20-methylcholanthrene would have more than the minimum tumor-producing dose; hence this concentration was not tested in this experiment. Pellets containing 1, 0.1, 0.01 and 0.001 per cent methylcholanthrene were implanted in 4 groups of 10 mice each.

Sarcomas developed between the 5th and 9th months in 4 of the mice that had received 1 per cent pellets. No sarcomas were produced in any of the other mice in this experiment. However, in the mice that had received pellets containing 0.001 per cent methylcholanthrene hemangiomas developed at or near the pellet in 2 mice. In 1 of these the hemangioma was directly in contact with the pellet; in the other the hemangioma was a few millimeters from the pellet. In a 3rd mouse in this group, in which no pellet was found at autopsy, a hemangiomatous lesion developed at a site where the pellet might possibly have been.

Cholesterol Pellets

As a control, cholesterol pellets of the same size, made in the same way except that no hydrocarbon was added, were implanted in 30 mice. No tumors were obtained in any of these mice.

Other Observations

Mammary gland tumors developed in 11 of the mice; these animals were distributed as follows: 4 and 1 in the groups receiving 5 and 0.001 per cent dibenzanthracene pellets, respectively; 1 and 2 in the groups receiving 1 and 0.1 per cent benzpyrene pellets, respectively; and 3 in the group receiving 0.001 per cent methylcholanthrene pellets. In all but 1 animal the mammary gland tumors arose at sites sufficiently distant from the pellets so there appeared to be no reason for assuming any association between the tumors and the pellets. The one exception occurred in the 5 per cent dibenzanthracene group; in this mouse the mammary gland tumor was found surrounding the pellet. Since this association may have been fortuitous, and since this tumor may have been a spontaneous one, it has not been included in the table.

A striking picture of generalized lymphomatosis was noted in

only 2 mice, 1 of which had received a 0.01 per cent benzpyrene pellet, and the other a 0.001 per cent methylcholanthrene pellet.

The fate of the pellets was essentially the same as in the previous investigation with dibenzanthracene-cholesterol pellets. In the majority of the mice the pellets were recovered intact at autopsy. In some, however, the pellets were softened, in others they were semifluid, and in a few instances they were much smaller than initially. In 1 mouse the pellet was seen in the process of extrusion through the skin. In the mice in which no pellet was seen at autopsy the pellet may either have been extruded unnoted, or may possibly have been absorbed.

Discussion

The purpose of these experiments was to block out the concentration ranges which would be in the vicinity of the minimum required for the regular production of tumors with this hydrocarbon-cholesterol pellet technic. In agreement with the results Shear,[1] and Shear and Lorenz [8] obtained with dibenzanthracene-cholesterol pellets in strain A mice, concentrations of hydrocarbon below 1 per cent were usually ineffective (see Table I). For pellets weighing from 7 to 60 mg. a concentration of 1 per cent would be equivalent to a hydrocarbon content of 0.07 to 0.6 mg.

The unexpected occurrence of 2 hemangiomas and 1 hemangiomatous lesion in 3 of the 10 mice that had received 0.001 per cent methylcholanthrene pellets raises the question as to whether they were induced or spontaneous. Clarification of this point requires further work with a larger number of mice injected with 0.001 per cent pellets of this hydrocarbon.*

However, this finding is exceptional, as was the development of a sarcoma in 1 of the 18 strain A mice which had received 0.001 per cent dibenzanthracene-cholesterol pellets; this mouse had received a pellet weighing 39.8 mg. and containing 0.4 gamma of the hydrocarbon (Shear,[1] and Shear and Lorenz [8]). Dobrovolskaïa-Zavadskaïa [9] has recently reported obtaining 2 sarcomas in 167 mice, of unstated strain, in a period of 11 months following the subcutaneous injection of 2.5 gamma of dibenzanthracene

* Dr. Harold L. Stewart (unpublished data) obtained no tumors in 16 strain D male mice which had received 0.001 per cent methylcholanthrene-cholesterol pellets subcutaneously in the right axilla; 9 of the mice survived for 12 to 18 months.

dissolved in 1/40 cc. of olive oil. In the light of these results the production of a sarcoma by a cholesterol pellet containing 0.4 gamma of dibenzanthracene now appears to be less of an anomaly than formerly.

For the regular production of sarcomas in mice in the tissues contiguous with the pellets, concentrations somewhat higher than 1 per cent appear to be required. For pellets weighing about 10 mg. this would be equivalent to a hydrocarbon content somewhat higher than 0.1 mg. With higher concentrations of hydrocarbon, smaller pellets may be found preferable.

It is conceivable that more tumors would have developed had the experiment lasted longer than 11 months. However, in investigations in which the pellet technic is employed for the purpose of studying premalignant tissue changes, or of obtaining more precise data on the amount of hydrocarbon consumed in tumor genesis, it may be more desirable to use concentrations somewhat above the minimum rather than to extend the experimental period much above a year. In order to shorten the experimental period still further it might be preferable to employ C_3H mice, since Andervont [6] has found that hydrocarbons appear to induce tumors more rapidly in mice of this strain than in strain A or in strain D mice.

It may be noteworthy that very few instances of generalized lymphomatosis were noted in these 200 mice, although Morton and Mider [10] obtained an incidence of 20 per cent in strain D mice painted with methylcholanthrene in benzene solution. However, this wide difference may be due to the difference in technic, inasmuch as Andervont [5] obtained lung tumors in a smaller percentage of the strain A mice that had received dibenzanthracene in the form of cholesterol pellets than in the mice that had received the hydrocarbon in lard solution or in other forms of dispersion. It is conceivable that a carcinogenic hydrocarbon produces lymphomatosis in a high proportion of susceptible mice only when it is present in a form that enables it to come into effective contact with hemopoietic tissue.

Summary

1. Cholesterol pellets containing concentrations of carcinogenic hydrocarbons ranging from 0.001 per cent to 5 per cent were im-

planted subcutaneously in strain D mice. The experiment was terminated after 11 months.

2. No tumors were obtained at the site of injection of cholesterol pellets containing 0.1 per cent or less of 1:2:5:6-dibenzanthracene.

3. No tumors were obtained at the site of injection of cholesterol pellets containing 1 per cent or less of 3:4-benzpyrene.*

4. No tumors were obtained at the site of injection of cholesterol pellets containing 0.1 or 0.01 per cent of 20-methylcholanthrene. Of the 10 mice that had received pellets containing 0.001 per cent of this hydrocarbon hemangiomas developed in 2 and a hemangiomatous lesion in 1.

5. Pellets containing cholesterol alone produced no tumors.

NOTE: The authors are indebted to Dr. Harold L. Stewart for the histological diagnoses.

* In subsequent work in collaboration with Mr. Robert D. Sall of the Harvard Medical School 1 per cent benzpyrene-cholesterol pellets produced tumors in 4 of 20 strain A mice in 5 months.

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STUDIES IN CARCINOGENESIS *

X. Production of Tumors by 3:4-Benzpyrene in Rats Fed Diets Containing Different Levels of Vitamin A

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In studying the tissue changes that developed in rats as a consequence of vitamin A deficiency, Wolbach and Howe [1] were impressed with the greatly augmented growth activity of epithelium; in some animals the number of mitotic figures in the replacing epithelium and the response of the connective tissue and of the blood vessels were such as to suggest "the acquisition of neoplastic properties." Among the results of vitamin A deficiency in the guinea pig Wolbach and Howe [2] noted a striking enlargement of the uterus and an excessive growth of keratinizing epithelium in the bladder.

These observations raised the question as to whether a deficiency of vitamin A in the diet would promote the genesis of tumors by a hydrocarbon carcinogen. To explore this possibility benzpyrene was injected subcutaneously in 65 rats which were divided into groups receiving A-rich and A-poor diets, respectively. Tumors were obtained in the rats fed both types of diet; neither the incidence of tumors nor the length of the latent period gave indication of a promoting effect as a result of A-deficiency.

Experimental

5 Per Cent Benzpyrene-Cholesterol Pellets

Cholesterol pellets containing 5 per cent of 3:4-benzpyrene, made according to the method of Shear,[3] were implanted (11/13/35) subcutaneously in the anterior part of the left flank of 26 rats. The weights of the pellets ranged from 3.5 mg. to 55.5 mg. Seventeen of the animals were albino rats of Wistar stock; 9 were hooded rats of unstated origin. All the animals were about 6 weeks old.

* Received for publication December 1, 1939.

Up to the time of injection of the carcinogen the rats were fed Sherman and Campbell's [4] diet B (No. 13) and some liver and lettuce. After the injection the rats were divided into 2 groups of 13 each, with an approximately equal distribution as to sex and color. One group was given a diet rich in vitamin A while the other received an A-deficient diet; these diets were similar to those employed by Wolbach and Howe.[1]

TABLE I

Production of Subcutaneous Tumors by 3:4-Benzpyrene in Rats Fed Diets of Different Vitamin A Content

Rats			Form of carcinogen	Vitamin A in diet	Tumors produced	
Initial number	Origin	Age			Number of rats	Time
		wks.				
13	Wistar and hooded	6	5% pellets	High	8	Between 3 and 15 mos.
13	Wistar and hooded	6	5% pellets	Very low	0	(Poor survival)
7	Wistar	6	5% pellets	High	4	Between 5 and 8 mos.
8	Wistar	6	5% pellets	Low	4	Between 4½ and 7½ mos.
12	Market	Adult	5 mg. crystals	High	7	Between 4½ and 9 mos.
12	Market	Adult	5 mg. crystals	Low	10	Between 4½ and 9½ mos.

In the A-rich group 8 of the 13 animals developed tumors around the pellets between the 3rd and 15th months (see Table I). No tumors were obtained in the rats on the A-deficient diet, for the deficiency was so severe that the rats went downhill rapidly; most of the animals either died or had to be killed by the end of the 4th month. The pellets were recovered intact in all but 1 of these rats.

The experiment was thereupon repeated, but just enough carotene was administered daily to the rats fed the A-deficient diet to prevent them from dying off too early, inasmuch as in the first experiment the rats fed the A-deficient diet did not survive the latent period of tumor production for benzpyrene-cholesterol pellets. Accordingly, 5 per cent benzpyrene pellets were implanted (6/1/36) subcutaneously in 15 Wistar albino rats, of both sexes, about 6 weeks old. Seven rats were given the A-rich diet; the

other 8 were given the A-deficient diet supplemented with a small amount of carotene.

No evidence of a significant difference in response was noted. In the rats fed the A-deficient diet tumors developed around the pellets in 4 of the 8 rats between 4½ and 7½ months; in the A-rich group tumors developed around the pellets in 4 of the 7 rats between the 5th and the 8th months.

The pellets were recovered at autopsy in 9 of the 15 animals.

Crystalline Benzpyrene

At the same time that the second pellet experiment with young rats was started, each of 24 adult albino market rats received subcutaneously 5 mg. of crystalline 3:4-benzpyrene, moistened with glycerol for lubrication as in the experiments with mice (Shear [5, 6]). The rats were divided into 2 groups with an approximately equal distribution as to sex.

In the rats fed the A-deficient diet supplemented with a small amount of carotene, tumors developed around the pellets in 10 of the 12 rats between 4½ and 9½ months. In those fed the A-rich diet tumors developed around the pellets in 7 of the 10 rats that survived for 4½ months or longer; these tumors appeared between 4½ and 9 months.

There was thus no indication of a significant difference in response to the action of the benzpyrene as a result of the difference in the vitamin A content of the diets.

Discussion

Although epithelial hyperplasia and metaplasia have been shown (Wolbach and Howe [1, 2]) to develop in experimental animals as a consequence of A-deficiency, and although McCullough and Dalldorf [7] found that in rats A-deficiency was an essential for the production of epithelial metaplasia by theelin or by mechanical irritation, it has not been established that malignant changes can be produced by A-deficiency.

There are numerous contributions which deal with vitamin A in cancer; these are concerned with the vitamin A content of tumors, the effect of vitamin A on cultures of malignant tissue *in vitro*, and with the influence of variation in the A-content of

the diet on the establishment or regression of transplanted tumors and on the regression of spontaneous animal and human neoplasms. The influence of vitamin A on the genesis of spontaneous tumors has also been investigated (see Gordonoff and Ludwig [8] for the bibliography, to which may be added the reports of Sugiura and Benedict,[9] Harde,[10] and Harde and Kobozieff [11]). The results so far obtained have not established that this vitamin plays an important rôle in these respects.

Vitamin A has also been considered as possibly playing a rôle in the genesis of induced tumors. Later investigators were not able to confirm Fibiger's classic report on the production of malignant tumors in the forestomach of rats fed a parasite and white bread; they considered the diet to be of greater significance than the parasite. The changes in the stomach of the rat which were obtained in later investigations were believed, by some authors, to be neoplastic. These changes, whether malignant or not, have been variously ascribed to a deficiency of vitamin A or of some other dietary requirement (see Cramer [12] for discussion and bibliography, to which may be added the reports of Bisceglie,[13, 14] Sugiura and Benedict,[9] and Sharpless [15]). Passey and Woodhouse (Passey [16]) obtained no significant difference in response to painting with an extract of soot between 2 groups of mice fed A-rich and A-poor diets respectively. Deprivation of vitamin A during estrin treatment (Bonser [17]) neither facilitated tumor development in a susceptible strain of mouse nor induced it in a resistant one, although it did hasten the appearance of the squamous metaplasia which estrin sets up in the coagulating gland and increased the degree of keratinization to an enormous extent. According to Geréb,[18] an excess of vitamins A, B, C and D in the diet of mice painted with tar facilitated the growth of tumors, metastases and recurrences; a deficient intake had the opposite effect; while variations in the amount of the individual vitamins were without effect. Geréb noted no effect on tumor genesis.

It is conceivable that application of a carcinogen directly to the epithelial tissues which develop the above mentioned hyperplasia and metaplasia might produce carcinomas in such tissues more readily in animals fed an A-deficient than in ones fed an A-rich diet. As far as the genesis of tumors in other tissues is concerned, however, no evidence of a promoting effect produced by A-de-

ficiency has been obtained in these exploratory experiments with benzpyrene implanted in the subcutaneous tissues of rats.

Summary

1. 3:4-Benzpyrene was implanted subcutaneously into 65 rats which were then fed vitamin A-rich or A-poor diets.

2. Young rats, implanted subcutaneously with 5 per cent benzpyrene-cholesterol pellets and fed a markedly A-deficient diet, developed severe symptoms of A-deficiency and died during the latent period of tumor production.

3. When enough carotene was added to the A-deficient diet to permit the rats to survive the latent period, tumors developed both in young rats implanted subcutaneously with 5 per cent benzpyrene-cholesterol pellets and in adult rats implanted subcutaneously with crystalline benzpyrene.

4. No indication of a significant difference in response to this carcinogen was observed as a result of variation in the vitamin A content of the diet.

Note: The authors are indebted to Dr. S. B. Wolbach and to Dr. Harold L. Stewart for examining the histological material.

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STUDIES IN CARCINOGENESIS *

XI. Development of Skin Tumors in Mice Painted with 3:4-Benzpyrene and Creosote Oil Fractions

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with the technical assistance of
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Industrial experience with coal tar products for a period of more than 30 years has enabled one of us (S. C.) to make observations on the effect of coal tar products in producing dermatitis. It appeared that while the higher boiling fractions and residuals, which contained anthracene and related substances, were strikingly irritating to the skin of workmen exposed to these fractions alone, no appreciable irritation was noted when the lower boiling members of this series were also present. In one workman, exposure to material containing only the lower boiling fractions was followed by regression of a previously existing skin wart. The impression was gained that coal tar pitch or high boiling anthracene oil frequently produced irritation of the skin in workmen handling these materials, but that no appreciable irritation occurred when these materials also contained 10 per cent, or more, of the lower boiling fractions. These latter fractions contain most of the benzene and naphthalene derivatives, including their phenols.

The experiments described in this paper were designed to ascertain whether such low-boiling fractions would exert an inhibiting effect on the production of skin tumors in mice painted with 3:4-benzpyrene.

Materials and Methods

The various fractions were prepared from a creosote oil (designated as Fraction I) obtained from a vertical retort coal tar; 90 per cent of the creosote oil distilled between 160° and 300° C. The basic constituents (Fraction II) were removed with aqueous

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hydrochloric acid; the phenols (Fraction III) were removed with aqueous sodium hydroxide. The remaining neutral material (Fraction IV) was steam-distilled. About 70 per cent of this material was obtained in the distillate (Fraction V); the residue was designated as Fraction VI. These fractions, dissolved in benzene, were painted on the skins of mice to obtain information on their toxicity; the concentrations employed had approximately the same relative proportions which they had in the original creosote oil. Fractions V and VI were also tested without dilution with benzene.

After the preliminary tests for toxicity, solutions in benzene were prepared which contained 0.2 per cent of 3:4-benzpyrene in addition to the selected concentrations of the coal tar fractions. A duplicate set of solutions was prepared in which the benzpyrene concentration was 0.05 per cent. Female albino market mice, in groups of 20 animals each, were painted 3 times weekly with these solutions. The control groups were painted with solutions containing 0.2 and 0.05 per cent, respectively, of benzpyrene.

Before starting the painting the hair on the back was clipped short. The solutions were applied to the skin of the clipped areas by means of swabs, which were made by attaching a small amount of absorbent cotton to the end of a narrow, cylindrical wooden applicator. For each mouse the swab was dipped into the solution and the excess drained by pressing the swab lightly against the wall of the container; as a rule, five strokes, without redipping, were made with the swab on each mouse.

Painting with the solutions containing the creosote oil fractions and benzpyrene was begun 4/14/36, and was carried out 3 times weekly until 9/1/36 (20 weeks); from this time on, the solutions containing 0.2 per cent benzpyrene were applied only twice weekly until 10/14/36 (6 weeks), when all painting with this concentration of benzpyrene was discontinued. Thus, over a period of 26 weeks, the mice had been painted 72 times. With the solutions containing the lower concentration of benzpyrene, painting was continued until 1/6/37, for a total of 114 times over a period of 38 weeks. When a papilloma appeared, painting was continued until the skin growth attained an "average diameter" (Shear [1]) of about 4 mm.

The mice were examined weekly as a routine, and oftener on occasion. Each mouse was identified and individual records were

TABLE I

Tumor Production in Mice Painted with 0.2 Per Cent Benzpyrene and Creosote Oil Fractions

Group*	Creosote oil		Number of mice which developed tumors (+), and number of negative survivors (−), after the intervals shown (in weeks)														Tumor-bearing mice (total number)	Regressions‡
	Fraction	Concentration	12	13	14	15	16	17	18	19	20	22	24	26	28	32	36	
		%																
I b	Unfractionated	33.3	..	..	1+	..	.	2+	..	..	1+	..	3+	2+	1+	1+	2+	13	2
			..	..	18−	..	.	16−	..	..	14−	..	8−	6−	5−	4−	2−		
II b	Basic	1.0	3+	..	2+	3+	..	1+	..	..	3+	1+	3+	3+	..	1+	..	20	0
			17−	..	15−	12−	..	11−	..	..	8−	7−	4−	1−	..	0−	..		
III b	Phenolic	6.6	..	..	..	2+	..	1+	1+	2+	..	4+	5+	..	..	3+	..	18	0
			..	..	..	18−	..	17−	16−	14−	..	9−	4−	..	..	0−	..		
IV b	Neutral	25.0	1+	1+	..	2+	..	1+	..	..	1+	2+	6+	2+	..	..	..	16	0
			19−	17−	..	15−	..	12−	..	..	11−	9−	3−	0−	..	..	..		
V b	Neutral distillate	25.0	1+	..	1+	1+	1+	1+	..	2+	..	7+	2+	1+	2+	..	1+	20	0
			19−	..	18−	17−	16−	15−	..	13−	..	6−	4−	3−	1−	..	0−		
V d	Neutral distillate	99.8	..	..	..	..	..	..	..	..	1+	..	..	..	..	1+	3+	7†	4
			..	..	..	..	..	..	..	..	16−	..	..	..	..	9−	5−		
VI b	Neutral residue	99.8	..	1+	..	..	..	..	..	2+	1+	1+	3+	1+	1+	3+	1+	14	1
			..	19−	..	..	..	..	..	17−	15−	14−	11−	9−	8−	5−	4−		
VII b	**	..	..	..	1+	2+	3+	3+	3+	1+	3+	..	..	2+	1+	..	..	19	0
			..	..	18−	16−	13−	10−	7−	6−	3−	..	..	1−	0−	..	..		

* 20 mice in each group.

** Control (0.2% benzpyrene).

† Tumors appeared in 2 mice after 37 weeks.

‡ Number of mice in which papillomas regressed without subsequent development of progressively growing tumors; these mice were not included, in the preceding columns, as tumor-bearing mice.

TABLE II

Tumor Production in Mice Painted with 0.05 Per Cent Benzpyrene and Creosote Oil Fractions

Group*	Creosote oil		Number of mice which developed tumors (+), and number of negative survivors (−), after the intervals shown (in weeks)															Tumor-bearing mice (total number)	Regressions‡
	Fraction	Concentration	21	22	23	24	25	26	27	28	30	32	36	40	44	48	52		
		%																	
I c	Unfractionated	33.3	..	..	..	..	..	..	..	..	..	..	2+	2+	1+	..	3+	8	3
			..	..	..	..	..	..	..	..	..	..	16−	14−	12−	..	7−		
II c	Basic	1.0	..	1+	1+	1+	2+	..	1+	..	3+	5+	4+	1+	..	..	..	19	0
			..	19−	18−	17−	15−	..	13−	..	10−	5−	1−	0−	..	..	..		
III c	Phenolic	6.6	..	..	..	..	..	..	..	..	1+	..	4+	4+	2+	..	1+	12	2
			..	..	..	..	..	..	..	..	19−	..	15−	11−	8−	..	6−		
IV c	Neutral	25.0	1+	1+	1+	1+	..	3+	2+	2+	2+	2+	..	2+	1−	..	..	18	0
			18−	17−	16−	14−	..	11−	9−	7−	5−	3−	..	1−	0−	..	..		
V c	Neutral distillate	25.0	1+	..	..	..	..	..	..	3+	2+	3+	6+	1+	1+	..	..	17	0
			19−	..	..	..	..	..	..	15−	13−	10−	3−	2−	1−	..	..		
V e	Neutral distillate	99.95	..	..	..	..	..	..	..	..	..	1+	1+	..	..	..	0+	5†	3
			..	..	..	..	..	..	..	..	..	17−	15−	..	..	..	14−		
VI c	Neutral residue	99.95	..	..	..	..	..	..	..	..	2+	..	4+	1+	3+	..	1+	11	6
			..	..	..	..	..	..	..	..	15−	..	10−	8−	4−	..	3−		
VII c	**	..	..	..	1+	..	..	..	..	2+	1+	2+	4+	5+	1+	..	..	16	1
			..	..	17−	..	..	..	..	15−	14−	12−	7−	1−	0−	..	..		

*20 mice in each group.
** Control (0.05% benzpyrene).
† Tumors developed in 3 mice during the 13th month.
‡ Number of mice in which papillomas regressed without subsequent development of progressively growing tumors; these mice were not included, in the preceding columns, as tumor-bearing mice.

kept for each. The histological material obtained at autopsy will be described in a later communication.

Not all of the papillomas which appeared on the painted areas developed into large tumors. In a significant number of cases the papillomas were sloughed off; other papillomas developed later in many instances. In this report the designation "tumor" is restricted to those neoplasms which grew progressively and did not regress. In comparing the rates of production of skin tumors in the different groups, the time of first appearance of the papillomas which continued to grow progressively until death occurred is given in the tables and charts. The mice in which papillomas regressed without subsequent development of progressively growing tumors were not considered as tumor-bearing mice in these comparisons.

Results

Toxicity Tests

In the preliminary tests for toxicity, groups of 5 mice each were painted 3 times weekly with the solutions having the composition described in the tables, except that they contained no benzpyrene. When evidence of lethal or severe toxic effects was not noted after 7 weeks, the experiments with the benzpyrene-containing solutions were begun.

The painting with the benzpyrene-free solutions was continued, however, for the duration of the investigation (38 weeks). No tumors were produced by any of these solutions except for solution VI, which produced papillomas in 1 mouse after 9 months; these papillomas grew very slowly and were not malignant when the mouse was killed 36 weeks after their first appearance. This solution consisted entirely of Fraction VI, with no added benzene or benzpyrene; it was the residue of the neutral fraction remaining after distillation of Fraction V, and is the fraction that would be likely to contain whatever hydrocarbon carcinogen might be present in the creosote oil.

Benzpyrene Control Solutions

For the control solution it was considered desirable to use a concentration of benzpyrene which would be high enough to produce tumors in a large proportion of the mice, and yet not so high

that any retarding effect of the test materials would be masked. A concentration of 0.05 per cent benzpyrene was selected for this purpose. However, since it appeared possible, at the time these experiments were begun, that this concentration might be too low, a concentration of 0.2 per cent benzpyrene was also employed.

The results are summarized in the tables and charts, in which the 0.2 and 0.05 per cent benzpyrene control experiments are designated VII b and VII c, respectively. With the higher concentration of benzpyrene, tumors were obtained in 19 of the 20 mice; 16 of these tumors developed in 22 weeks, at which time no tumors had yet been obtained with the lower concentration (compare Curves VII b and VII c in Charts 1 and 2). With 0.05 per cent benzpyrene, tumors were obtained in 16 mice; a papilloma that developed after 25 weeks in a 17th mouse later regressed.

Retarding Solutions

In spite of the pronounced difference in potency of the two concentrations of benzpyrene, the solutions that exhibited a retarding

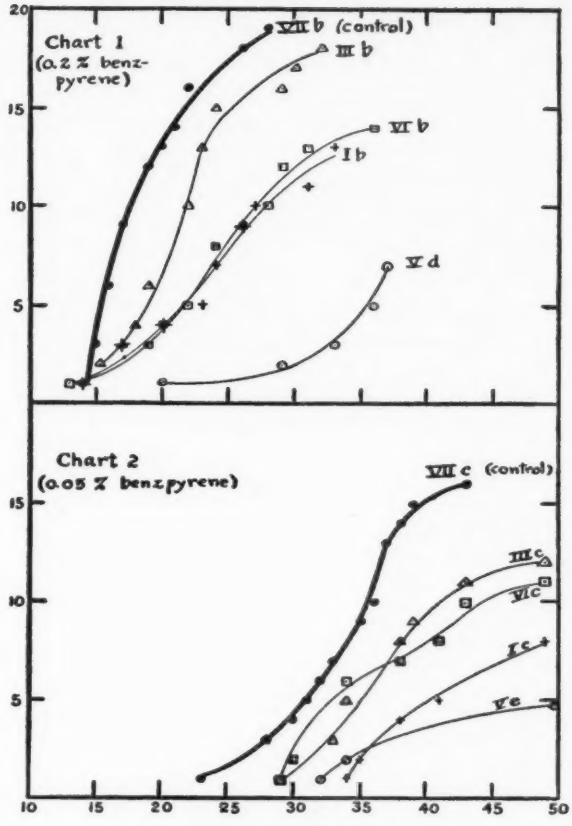
TABLE III

Creosote Oil Fractions which Retarded Tumor Production

Solution	Composition			Solvent
	Benzpyrene	Creosote oil fraction		
	%		%	
III b	0.20	Phenolic	6.6	Benzene
III c	0.05	Phenolic	6.6	Benzene
VI b	0.20	Neutral residue	99.8	....
VI c	0.05	Neutral residue	99.95	....
I b	0.20	Unfractionated	33.3	Benzene
I c	0.05	Unfractionated	33.3	Benzene
V d	0.20	Neutral distillate	99.8	....
V e	0.05	Neutral distillate	99.95	....

effect on tumor production by 0.05 per cent benzpyrene gave entirely analogous results in the experiments with 0.2 per cent benzpyrene. Four of the test solutions delayed tumor production; the results of these experiments are summarized in Charts 1 and 2, in which the heavy, full lines (Curves VII b and VII c) represent the benzpyrene control experiments.

In the order of increasing retardation the solutions and their composition are shown in Table III.



RETARDING EFFECT

Rate of production of skin tumors in mice painted with solutions containing fractions of creosote oil.

CHART 1. *All solutions contained 0.20 per cent benzpyrene.*

III b =	6.6% phenol fraction	plus	0.20%	benzpyrene
VI b =	99.8% neutral residue	"	"	"
I b =	33.3% unfractionated oil	"	"	"
V d =	99.8% neutral distillate	"	"	"
VII b =	(control)		"	"

CHART 2. *All solutions contained 0.05 per cent benzpyrene.*

III c =	6.6% phenol fraction	plus	0.05%	benzpyrene
VI c =	99.95% neutral residue	"	"	"
I c =	33.3% unfractionated oil	"	"	"
V e =	99.95% neutral distillate	"	"	"
VII c =	(control)		"	"

Damage to the skin, consisting of denudation of hair, reddening, induration and some ulceration, occurred in many of the mice. The extent of this damage was greatest in the mice in groups I b, V d and VI b. Very much less damage to the skin was produced in groups I c, V e and VI c, although these received the same concentrations of creosote oil constituents as I b, V d and VI b.

The retardation produced by the phenolic fraction (III b and III c) did not, however, appear to be attributable to obvious skin damage. In fact, remarkably little visible skin damage was produced by III c; no more evidence of damage was noted in this group than in II c, which exhibited a promoting effect on tumor production.

Promoting Solutions

With the higher concentration of benzpyrene, 3 of the solutions gave results which paralleled closely those given by the control

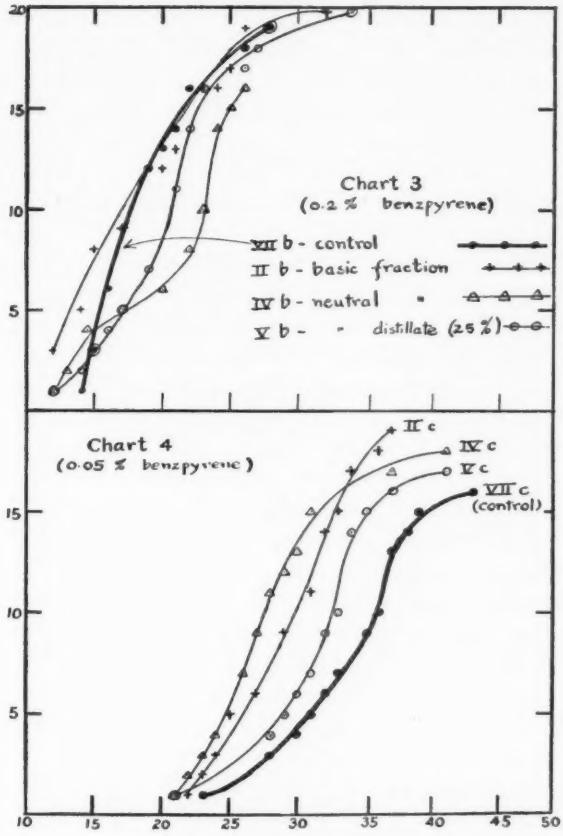
TABLE IV

Creosote Oil Fractions which Accelerated Tumor Production

Solution	Composition			Solvent
	Benzpyrene	Creosote oil fraction		
	%		%	
II c	0.05	Basic	1.0	Benzene
II b	0.20	Basic	1.0	Benzene
IV c	0.05	Neutral	25.0	Benzene
IV b	0.20	Neutral	25.0	Benzene
V c	0.05	Neutral distillate	25.0	Benzene
V b	0.20	Neutral distillate	25.0	Benzene

solution (see Chart 3). With the lower concentration of benzpyrene, however, these 3 solutions produced tumors more rapidly than did the control solution (see Chart 4). The composition of these solutions is shown in Table IV.

The amount of benzpyrene at the higher concentration was apparently sufficiently great to mask the promoting effect which was more evident at the lower benzpyrene concentration. Examination of the curves in Chart 3 reveals, however, that all 3 of these solutions began to produce tumors earlier than the control. This was most pronounced in the case of the basic fraction (see



PROMOTING EFFECT

Rate of production of skin tumors in mice painted with solutions containing fractions of creosote oil.

CHART 3. *All solutions contained 0.20 per cent benzpyrene.*

II b =	1% basic fraction	plus	0.20%	benzpyrene
IV b =	25% neutral fraction	"	"	"
V b =	25% neutral distillate	"	"	"
VII c =	(control)		"	"

CHART 4. *All solutions contained 0.05 per cent benzpyrene.*

II c =	1% basic fraction	plus	0.05%	benzpyrene
IV c =	25% neutral fraction	"	"	"
V c =	25% neutral distillate	"	"	"
VII c =	(control)		"	"

Curve II b) which, in 15 weeks, produced tumors in 8 mice; in the control group (VII b) only 3 mice bore tumors at this time.

Discussion

The retarding effect produced by solutions V d and V e appears to be attributable to the skin damage produced, rather than to a specific inhibitory effect, for when the concentration of the neutral distillate was reduced from 100 to 25 per cent (solutions V b and V c) the damage to the skin was less and no retardation was noted. Whether the slightly more rapid tumor production by V c, as compared with the control solution (VII c), represents a significant difference cannot be deduced from these data.

The entire creosote oil (I b and I c) and the neutral residue (VI b and VI c) also retarded tumor production but here, too, damage to the skin was greater than in the corresponding control mice. Moreover, the concentrations of the fractions employed were high (33.3 and 100 per cent, respectively).

The retarding effect obtained with the phenolic fraction (III b and III c) appears to be of greater interest for two reasons. In the first place, little skin damage was noted and, in the second place, the comparatively low concentration of 6.6 per cent was used. At the end of 43 weeks tumors had been produced in only 11 mice of group III c as compared with tumors in 16 of the control mice (see Chart 2). This was not due to higher mortality in this test group, since 8 mice in III c were still alive and negative at 43 weeks, whereas there were no negative survivors in the control group at this time (see Table II).

In further investigations of the retarding action of such material it would appear to be preferable to use concentrations of benzpyrene in the vicinity of 0.05 per cent rather than 0.2 per cent, for the benzpyrene itself contributes to the damage to the skin which is greater the higher the benzpyrene concentration. It would, moreover, be of interest to employ lower concentrations of the other fractions, particularly of the phenolic fraction. Apart from the possible value of such information for the prevention of dermatitis and skin cancer in workmen in the coal tar and related industries, it would be of interest for a better understanding of the mechanism of the production of skin tumors to ascertain

whether any coal tar constituents act as "anti-carcinogens" (Berenblum [2]).

The basic fraction exhibited a promoting action at both levels of benzpyrene. This is the only one of these creosote oil fractions that it has so far been feasible to investigate further. In subsequent painting experiments,* in which strain A mice and solutions containing 0.05 and 0.02 per cent benzpyrene were employed, skin tumors developed in mice painted with solutions containing 2 per cent of the basic fraction and benzpyrene more rapidly than in the control mice painted with benzpyrene alone. Other control mice, painted with a solution containing only 2 per cent of the basic fraction, developed no skin tumors.

Experiments have also been carried out to ascertain whether an analogous promoting effect would be obtained in the production of subcutaneous tumors by benzpyrene. In mice injected with 0.5 mg. of benzpyrene in lard containing 2 per cent of the basic fraction, tumors did not develop any more rapidly than in mice that received the benzpyrene alone, but even the control mice developed tumors quite rapidly. When the dose was reduced to 0.1 mg. of benzpyrene, however, subcutaneous tumors developed, slowly, in 45 per cent of the control mice, as contrasted with rapid development of tumors in 95 per cent of the mice which had received, in addition, the basic fraction.

This line of inquiry has been extended to other materials and individual compounds in tests of possible "co-carcinogenic" activity (Shear [3, 4]) and to other carcinogens. The results obtained so far have been complicated by what appears to be a contributory, and variable, action of different batches of the lard, used as solvent, upon the rate of tumor production. Other experiments are in progress [5, 6] in which tricaprylin is being employed as the solvent.

Summary

1. Albino market mice were painted with benzene solutions containing various fractions of creosote oil together with 0.2 and 0.05 per cent, respectively, of 3:4-benzpyrene.

2. The 0.05 per cent benzpyrene control solution began to produce skin tumors after most of the mice painted with the 0.2 per cent benzpyrene control solution already bore tumors.

* In collaboration with Mr. Robert D. Sall of the Harvard Medical School.

3. Four of the test solutions exhibited a retarding effect on tumor production. In 3 of these the retardation may have been due to damage to the skin.

4. The phenolic fraction, in a concentration of 6.6 per cent, exhibited a retarding effect which was not due to obvious skin injury.

5. Three of the test solutions exhibited a promoting effect. The basic fraction, in a concentration of 1 per cent, exhibited an accelerating effect at both benzpyrene levels.

NOTE: The authors are indebted to Dr. S. B. Wolbach for his interest and advice.

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THE PREPARATION OF TRICAPRYLIN *

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Tricaprylin has been found in this laboratory [1, 2, 3] to be a useful vehicle for the introduction of pure organic compounds into experimental animals. As pointed out by Fieser,[4] the substance is a single chemical individual unlike the commonly employed lard and other fats, and is therefore of uniform chemical properties and reproducible biological effects. In addition it bears a close chemical similarity to the saturated fats so widespread in plant and animal organisms, and may, indeed, occur as a minor constituent in such material. At least caprylic acid is known to occur as glyceride (whether as triglyceride is not known) in a wide variety of plant and animal fats and oils.

With the recent appearance on the market of a good commercial grade of caprylic acid at low cost,† it was believed that the publication of a process for the preparation of pure tricaprylin utilizing this acid would serve a useful purpose in making this substance more easily obtainable in quantity.

HISTORICAL

While caprylic acid was recognized as occurring as a glyceride in cow's butter as early as 1844,[5] and in cocoanut oil the following year,[6] it was not until 1899 that the synthesis of tricaprylin, from caprylic acid and glycerol, was reported by Scheij.[7] Scheij's method of heating together the reactants was followed, with modification, by all later investigators.[8–11] It seemed to me that a method for the synthesis of tricaprylin, utilizing the acid chloride as an intermediate step, would offer the advantages of more complete esterification of the polyalcohol and greater flexibility in the adaptation to the preparation of other glycerides, both simple and mixed. Another consideration is that caprylic acid chloride will probably soon become commercially available, and the purification

* Received for publication December 1, 1939.

† Sold by Armour and Company, Chicago, Ill., under the name "Neo-Fat No. 7."

of this material offers certain advantages over the purification of the acid.

Following several investigators,[12, 13] the thionyl chloride method of preparing the acid chloride was selected in preference to the other methods by which it has also been prepared. The complete procedure follows.

Procedure *

Caprylic Acid Chloride: Throughout this procedure the use of standard taper, all glass apparatus is desirable on account of the corrosive nature of the chemical substances to be handled.

Into a 3 liter round bottom flask fitted with a reflux condenser provided with a large enough exit to accommodate the evolution of large quantities of sulfur dioxide are placed 1 kg. of caprylic acid (commercial grade, melting point 13.0–14.0° C.) and 1650 gm. of thionyl chloride (good commercial grade, not subsequently purified). The mixture becomes cool and heat is applied gradually to avoid too violent a production of sulfur dioxide. The mixture is refluxed for 1.5 hours, access of moisture being prevented after the greater part of the gas has come off by attaching a drying tube filled with calcium chloride to the exit tube of the condenser.

After removal of about 465 gm. of low boiling material (chiefly thionyl chloride) up to 85° C. at atmospheric pressure, the crude product is brought over under reduced pressure, all distilling under 110° C. at 18 mm. being retained. This fraction consists of the acid chlorides of all acids present (about 10 per cent being a mixture of caproic and capric acid chlorides). When this crude cut is carefully fractionated through a Widmer column 661 gm. (59 per cent) of pure colorless material boiling at 97–98° C. (28 mm.) is obtained. As a test of the purity of this product a sample was hydrolyzed to the acid and the melting point of the latter determined; a value of 15.8–16.2° C. was found (best in the literature, 16.5° C.).

Tricaprylin: Into a 5 liter round bottom flask equipped with reflux condenser are placed 1 kg. of pure caprylic acid chloride, 2300 cc. of dry carbon tetrachloride and 172 gm. of pure anhydrous glycerol. To the mixture is slowly added 470 gm. of pyridine (technical grade, but dried over potassium hydroxide). A con-

* Acknowledgement is made of technical assistance by Mr. Joseph Leiter.

siderable amount of heat is evolved and an ice-bath is applied to the flask if ebullition is too vigorous. The contents of the flask are refluxed 18 hours. The white precipitate is presumaby pyridine hydrochloride. Solvent (about 1600 cc.) is removed until the mixture becomes too thick for further heating. Water (2 liters) is added to dissolve the solid. The carbon tetrachloride layer containing the product is separated off, the pyridine-acid chloride complex decomposed by extraction with two 1 liter portions of cold 5 per cent sulfuric acid. After washing once with water and several times with sodium bicarbonate solution to remove free caprylic acid (any emulsion at this point can be broken by adding water), the product is dried over anhydrous sodium sulfate. On distillation at atmospheric pressure an additional 550 cc. of carbon tetrachloride is recovered. After the remainder of the volatile material has been removed under the water pump, fractionation at 1 mm. yields a light yellow oil, boiling point 237–246° C.; yield 734 gm. (76 per cent based on acid chloride; 84 per cent based on glycerol) melting point 8.3–9.0° C. A forerun of about 105 gm. boiling up to 237° C. (1 mm.) consists chiefly of lower glycerides of caprylic acid and can undoubtedly be further treated with caprylic acid chloride to yield an additional quantity of tricaprylin.

If a colorless product be desired, the yellow oil may be mixed with an equal volume of dry ether and refluxed with decolorizing charcoal until decolorization is complete. After filtration, the ether is removed (finally under vacuum) and a satisfactory product is obtained having the same melting point as before. Optionally this colorless material may be distilled at the pressure of the mercury vapor pump (about 10^{-3} mm.) without acquiring color.

Summary

1. A method is described for the preparation of pure tricaprylin from commercially available chemicals of technical quality.

2. The preparation of pure caprylic acid chloride and its condensation with glycerol in the presence of pyridine are described.

Note: While this paper was in press a note on the preparation of tricaprylin by E. B. Hershberg appeared in *J. Am. Chem. Soc.*, 1939, **61**, 3587–3588.

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METASTATIC NEUROBLASTOMA IN BONE PRESENTING THE EWING SYNDROME, WITH A DISCUSSION OF "EWING'S SARCOMA" *

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INTRODUCTION

In 1933 Colville and I [1] reported a case of tumour of the femur which had exhibited in every detail the characteristics generally accepted as diagnostic of "Ewing's sarcoma," but which postmortem study proved to be a clinically precocious metastasis from a small primary neuroblastoma of one adrenal gland. In view of this finding, our only personal experience with "Ewing's sarcoma" up to that time, we reviewed previous records of cases so diagnosed but could not find any in which the postmortem observations were adequately enough recorded to afford convincing proof of the primary nature of the bone tumours. On the contrary, in some of the recorded cases we found evidence suggesting the possible metastatic nature of the tumours.

The present paper describes the 2nd case with the classical Ewing syndrome to be seen at this hospital. Autopsy in this case also proved that the supposedly primary femoral tumour was a metastasis from a neuroblastoma, arising in this instance from the left lumbar sympathetic chain.

REPORT OF CASE

Clinical History: D. S., a girl 17 years of age, was first admitted to the Alfred Hospital in March, 1936. In December, 1934, she had injured the left lower limb at play; it had been painful for a few days but had not been incapacitating. In March, 1935, swelling of the thigh was first noticed, and this steadily increased. In the following November surgical exploration of the tumour was carried out in Launceston, Tasmania. A large, fusiform soft tumour was found enveloping the femur. A piece of this was removed and microscopic examination revealed a cellular, undifferentiated round celled growth devoid of any osteogenic characteristics.† The patient was advised to

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† I subsequently had the opportunity of examining this section and it showed a cellular tumour tissue similar to that seen later in the growths found at autopsy and described below.

come to Melbourne and was referred for treatment to the Alfred Hospital, with a diagnosis of sarcoma of the left thigh.

Physical examination on admission showed a firm fusiform enlargement of the shaft of the left femur but no other physical abnormalities. A skiagram was reported as showing "evidence of periostitis along the lateral and medial aspects of the lower and middle thirds of the femur." Skiagrams of the lungs showed no signs of any metastatic growths. X-ray therapy applied during March produced prompt and complete subsidence of the swelling and the patient was discharged from the wards to attend the hospital as an outpatient. In November, 1936, she was well and at work; her weight was increasing, and the thigh remained normal in size. In April, 1937, re-enlargement of the thigh was noticed. A further course of X-ray treatment was given and again the tumour subsided. In June, 1937, during a routine examination, a large, smooth rounded mass about 15 cm. in diameter was unexpectedly discovered in the lower abdomen. This was fixed and evidently retroperitoneal in position, but it was painless and had caused the patient no inconvenience. During June and July a course of X-radiation was applied to this area. By October the mass had decreased in size, and by December it had almost disappeared. At this time the patient returned to her home in Tasmania. She was contemplating marriage but was advised to defer this step for a time.

During the early months of 1938 information was received from her medical adviser in Tasmania that recurrence of the growth in the abdomen had taken place, that there was skiagraphic evidence of metastases in the lungs, and that the patient was losing weight and strength. She returned to Victoria and was readmitted to the Alfred Hospital in June. She had clearly lost weight, the abdominal mass had attained a large size, and skiagrams showed spherical metastatic deposits in the right lung. The femoral tumour appeared to be little altered. During July some therapeutic X-radiation was applied to the abdomen and chest, but with little or no benefit. In August and September the left lower limb became increasingly edematous and painful, and a mass of growth appeared in the left groin. This growth soon extended to involve the skin of the thigh and perineum. The patient became bed-ridden and death ensued on Nov. 14, 1938, at the age of nearly 20 years.

Postmortem Examination

The body was that of an emaciated girl with prominent abdominal enlargement, great edema of the lower limbs and of the lower part of the trunk, and large irregular projections of growth from the skin of the left groin and upper half of the thigh. On dissection these were found to be continuous with deposits of white growth in the inguinal and iliac lymph glands and to fuse in the upper adductor region with the tumour in the femur. This was a fusiform growth involving the entire shaft of the femur, elevating the periosteum, extending diffusely into surrounding muscles in places, and when stripped from the bone it left an eroded bone surface like coarse sand-paper. There was also wide-

spread infiltration of the medullary cavity by growth. Figure 1, A, B and C, shows skiagrams of the femur and enveloping growth after removal from the body: new formation of bone has taken place in the form of both "onion-skin" layers parallel with the surface and spines vertical to the surface.

The main cause of the abdominal enlargement was a spherical retroperitoneal tumour 14 cm. in diameter lying anterior to the three lower lumbar vertebrae, with its centre slightly to the left of the midline. This tumour consisted of a peripheral zone of fibrosed growth varying in thickness from 0.5 to 2.5 cm., and a central mass of necrotic, discoloured, partly liquefied débris. Areas of gritty calcification were present in parts. The tumour was smooth and well defined over most of its surface but was continuous with a mass of growth in the left anterior part of the body of the fourth lumbar vertebra; it was also partially fused cranially and caudally with tumour deposits in the lumbar and iliac lymph glands. The left ureter was superficially incorporated in the posterior aspect of the tumour, and the left sympathetic chain entered and was lost in it. Figure 2 shows the distribution and relationships of the femoral and retroperitoneal tumours, and of the lymph glandular metastases associated with them. The deposits in the lymph glands consisted of uniform soft white growth and were extensively fused with one another and individually ill-defined.

The peritoneum presented widespread nodules and plaques of soft white growth, which clothed the pelvic organs as an almost diffuse sheet, filled the pouch of Douglas, and invaded the pelvic floor. The posterior wall of the bladder was invaded by the tumour. More discrete deposits were present on the omentum, mesentery, and the parietal and diaphragmatic peritoneum. Bilateral hydronephrosis and hydroureter were present, but the kidneys were otherwise normal. Save for the peritoneal deposits of tumour, the liver, spleen, pancreas, stomach, intestines and adrenals were normal.

The lungs contained a few, rounded, discrete metastatic growths, the largest of which was 3 cm. in diameter. The heart, thymus, trachea, oesophagus, thyroid, larynx, pharynx, oral and nasal cavities, cervical and mediastinal lymph glands, brain, meninges and pituitary gland were all normal.

In addition to the state of the left femur and of the fourth lumbar vertebra already described, the skeleton showed the following abnormalities. The right femur contained a single, small white metastasis situated in pink marrow in the middle of the shaft. The heads of several ribs presented small, film-like subperiosteal deposits. On the inner aspect of one parietal bone there was a small subperiosteal plaque of white, semifluid tumour tissue which, when wiped off, left a slightly eroded, granular bone surface. No tumour was found in the following bones on section: base of skull, thoracic vertebrae, lumbar vertebrae other than the fourth, sternum, several ribs and both iliac crests. The remaining bones of the skeleton were not sectioned.

Histology of the Tumours

Paraffin sections were prepared from many parts of the growths and were stained by haemalum and eosin, and by Heidenhain's iron haematoxylin and Van Gieson's stains. In all situations the healthy parts of the growths had a very similar structure, consisting of closely aggregated, undifferentiated, rounded or polyhedral cells, between 12 and 15 μ in average diameter, with scanty cytoplasm and rather indefinite cell outlines, and each with a single, rounded or ovoid nucleus 8 to 12 μ in diameter. In most situations the cells were devoid of any definite arrangement, forming irregular diffuse masses and sheets. In some parts of the large cystic retroperitoneal tumour, however, there were distinct "rosettes," appearing in section as non-nucleated rounded areas occupied by a tangle of fine fibrils, measuring between 30 and 50 μ in average diameter and surrounded by a ring of between fifteen and thirty cells (Figs. 3 and 4). It was only in parts of the large cystic lumbar growth that rosette formation was distinct; in all other growths the cells showed at most only vague suggestions of rosette grouping.

In view of the presence of rosettes identical in appearance with those of neuroblastoma, sections from more than fifty blocks from different parts of the retroperitoneal growth were searched carefully for the possible presence of well differentiated ganglioneuromatous areas, but without success. All but the peripheral parts of this tumour, however, had suffered degeneration with patches of calcification and extensive liquefaction, so that even had gan-

glioneuromatous areas been originally present, they might long since have disappeared. The left ureter and the left lumbar sympathetic chain were incorporated in the thick fibrosed periphery of the growth, and some sympathetic branches were found traversing the tumour.

Discussion

The Nature and Origin of the Tumour

The clinical diagnosis in this case had been "sarcoma of the femur," and the patient had presented perfectly the syndrome said to be characteristic of a "Ewing's tumour" of bone. The patient was an adolescent, the tumour was fusiform, involving a large part of the shaft of the bone diffusely, early skiagrams suggested "periostitis," later skiagrams showed "onion-skin" laminae and vertical spicules of new bone, biopsy section disclosed an undifferentiated round celled growth devoid of osteogenic characteristics, the growth was highly radiosensitive and was controlled effectively by X-ray treatment for over 2 years, and the autopsy disclosed metastases in lymph glands, lungs, and in other bones. In all respects, then, the case displayed the characteristics of "Ewing's tumour," as originally described by Ewing (1922,[2] and in his book entitled Neoplastic Diseases[3]), Connor (1926), Kolodny (1927)[4] and Copeland and Geschickter (1930).[5] In view, however, of the previous case at this hospital in which a "Ewing's tumour" of the femur was shown at autopsy to be a metastasis of an adrenal neuroblastoma (see Colville and Willis[1]), a suspicion that the present tumour might prove to be of a similar nature was entertained, and accordingly the designation of "Ewing's tumour" was avoided pending postmortem investigation. The fact that a large retroperitoneal mass became palpable during the course of the illness strengthened this suspicion.

The gross autopsy diagnosis was uncertain, but it was clear that the primary growth must be either (*a*) the tumour in the femur, or (*b*) the large cystic retroperitoneal tumour ("T" in Fig. 2). The first view involved the assumption that the tumour "T" was an unusually large and degenerated metastatic growth in the lumbar lymph glands with an unusually thick, fibrous outer wall. The second view involved the assumption that the femoral tumour

was an unusually precocious metastasis from the retroperitoneal growth.

Histological study of the autopsy material, in addition to the other observations, led to the confident conclusion that the tumour "T" in Figure 2 was a primary neuroblastoma of the left lumbar sympathetic chain, and that the femoral growth was indeed metastatic. The evidence in favour of this conclusion may be briefly summarized as follows: (1) The histology of the tumour was identical with that of known cases of neuroblastoma, both as regards the cell characteristics and the nature of the rosettes (see Figs. 3 and 4). As with some of the more malignant cases of neuroblastoma, such as that reported by Colville and me, distinct rosettes were seen only in the presumed primary tumour and not in the metastases. (2) The characteristics of the tumour "T" differed decidedly from those of the numerous metastatic growths in the neighbouring lymph glands. It was much larger than any of these; it was the only growth to show extensive degeneration and calcification; it had a thick, tough peripheral layer which made it a well defined structural entity amid the ill defined confluent secondary growths around it, and, also unlike these, it invaded the adjacent lumbar vertebra. (3) The position and relationships of the tumour "T" conformed with its presumed origin in the left lumbar sympathetic chain; it lay slightly to the left side, the sympathetic chain and the ureter were incorporated in it, and it had invaded the adjacent bone. These relationships are similar to those of many previously reported cases of lumbar or pelvic ganglioneuromas and neuroblastomas, references to which are given by McFarland and Sappington (1935),[6] and by Wahl and Craig (1938).[7] (4) The pathology of the femoral growth in the present case closely resembled that of the previous case of metastatic neuroblastoma of the femur reported by Colville and myself. Characteristics possessed in common by the two growths were the fusiform enlargement with expansion of the periosteum, the formation of "onion-skin" layers of periosteal new bone (compare Fig. 1 of the present paper with Fig. 1 of the previous case), a similar "sand-paper" surface of the bones when the soft tumour had been removed, infiltration of the marrow cavity, and diffuse invasion of surrounding soft tissues. The similarity of the morbid anatomy of the thigh in the 2 cases was

indeed very remarkable and afforded in itself strong presumptive evidence that the two tumours were of an essentially similar nature. It is to be noted that periosteal reaction, in the form of "onion-skin" laminae or vertical spicules of new bone, is frequently to be seen in the skeletal metastases of neuroblastomas (see Willis, 1938 [8]), and that Geschickter and Copeland (1936)[9] are in error in stating that such growths "are not accompanied by periosteal reaction." It is also worthy of emphasis that the multiplicity of bone involvement in the present case accords with the usual findings in cases of metastatic neuroblastoma. (5) It must also be noted that the age of the patient in the present case, nearly 20 years at her death, does not conflict with a diagnosis of neuroblastoma, and that Geschickter and Copeland's statement that neuroblastoma metastases in bone "occur at an extremely early age" expresses only a partial truth. Although it is true that most ganglioneuromas and neuroblastomas appear in young children, there are now many recorded cases of such tumours in adolescents and adults (see McFarland and Sappington, 1935,[6] and Wahl and Craig, 1938 [7]); and I, myself, have a specimen of widely metastasizing neuroblastoma from a man of 51 years, kindly given to me by Dr. Leila Hawksley of the Cancer Hospital, London, England.

On the evidence presented, the conclusion can scarcely be avoided that the case described was one of retroperitoneal neuroblastoma with a clinically precocious metastasis in the femur.

Ewing's Tumour of Bone

The observations recorded in the present paper strengthen the conclusions reached by Colville and me in 1933. It must surely be more than a mere coincidence that the only 2 cases of "Ewing's sarcoma" seen in this hospital should both prove, on careful post-mortem investigation, to be metastases from primary neuroblastomas elsewhere. Since our paper and my subsequent survey of the subject in 1934, many further reports of cases designated as "Ewing's tumor" have been published. The following brief review of some of these will show the unscientific confusion which still clouds the subject.

Connor, whose paper in 1926 maintained "Ewing's endothelial myeloma" to be an entity, states more recently (1934)[10]: "I am now certain that among the group of tumors reported by me in

1926 as Ewing's tumor, there were a number which were undoubtedly cases of multiple myeloma or lymphosarcomatosis with metastases to bones. The usual and just criticism has been that autopsies were not performed on most of the recorded cases, and that without a complete examination postmortem, a primary visceral tumor could not be excluded." Connor then reports (all too briefly) 3 autopsied cases in which he regards the diagnosis of Ewing's tumour as proved. His first case was a man of 31 years with a solitary scapular tumour without metastases; the tumour responded only slightly to X-radiation; the autopsy details are scanty, many organs not being mentioned, and the cause of a general peritonitis and great distention of the stomach is left unexplained. Connor's statement that the tumour "ought to be, by exclusion if for no other reason, an endothelial tumor of bone" is scarcely scientific evidence of its nature. In his 2nd case, a fibular tumour in a child of 6 years, with radiating spicules of new bone, in which X-radiation was of only "slight benefit," Connor admits that the tumour resembled osteogenic sarcoma, and he gives no information regarding the structure of the metastases which were present in the lungs and other bones. In his 3rd case, a femoral growth in a boy of 5 years, the growth responded to X-radiation; at autopsy growths were present also in the skull and lungs, the lower lobe of the right lung being entirely replaced by tumour; but the possibility that this large tumour might have been the primary one is not even referred to. Connor's conclusions speak for themselves: "From the three cases recorded here I believe we may say that the endothelioma of bone is much like the malignant endothelioma found elsewhere; that it does not respond rapidly and readily to X-ray treatment but disappears gradually and slowly as compared, for instance, with a metastatic lymphosarcoma or a neuroblastoma. . . . Another revision can be made with the evidence at hand. The tumor cell of Ewing's sarcoma can become an osteoblast and form bone." Since the two most generally accepted features of the Ewing syndrome have been the radiosensitive nature and non-osteogenic character of the growth, these conclusions of Connor add confusion to confusion.

Melnick (1933)[11] reports the case of a girl 15 years of age with a tumour of the femur, biopsy examination of which "was diagnostic of Ewing's sarcoma." (How can the microscopic ap-

pearance of an undifferentiated round celled growth be "diagnostic" of anything?) Autopsy revealed also an apical lung tumour, but all other viscera, lymph glands and bones were free from growth. Since, however, no details regarding examination of most of the organs are given, and since the response to X-radiation had been poor, the diagnosis in this case is questionable.

In the case of a tibial tumour in a boy of 5 years, reported by De Santo (1934),[12] the diagnosis of "Ewing's tumor — primary intracortical and subperiosteal lymphangio-endothelioma" was made on histological grounds. In addition to growths in many bones, autopsy revealed a large, upper lumbar retroperitoneal growth, and the possibility that this may have been primary is not given the consideration it deserves.

Stewart (1933)[13] diagnoses "endothelial myeloma" from microscopic examination of aspirated fragments, and he states that "on three occasions we have been able . . . to detect the small rosettes characteristic of this tumor." Since rosettes exactly like that depicted by Stewart are commonly present in neuroblastoma, how can they be "characteristic" of "endothelial myeloma"? In another paper Stewart[14] says: "Most endothelial myelomas are radiosensitive, but occasionally one sees a resistant endothelial myeloma"; while of the radiosensitivity of neuroblastoma, he says: "The retroperitoneal and suprarenal groups are highly sensitive." Clearly then, the criterion of radiosensitivity will not permit distinction between a "Ewing's tumour" and metastatic neuroblastoma, a point which is strikingly substantiated by the 2 cases reported by Colville and myself and in the present paper.

Coley (1935),[15] Hellner (1935),[16] Geschickter (1936)[17] and Bergstrand (1936)[18] refer to cases of "Ewing's sarcoma," but without autopsy report or any useful pathological information. Lattman (1934)[19] very briefly reports 3 cases of "Ewing's tumour"; only the 3rd case came to autopsy (of which only scanty details are given), and in this case no reasons are stated for discarding the diagnosis of "osteogenic sarcoma" made by both the pathologist and the radiologist during life.

Porter and co-authors (1936)[20] give a good account of a tumour of the pelvis in a woman aged 23 years. The autopsy revealed nodular growths in the lungs and pleura, and thick cords of growth parallel to the bronchi in all parts of the lungs. The authors con-

sider the possibility of a primary pulmonary carcinoma, but exclude this because of the absence of a massive tumour and of any obvious involvement of bronchial mucosa. They are apparently unaware of the infiltrating type of lung carcinoma which produces widespread peribronchial infiltration without any local tumefaction and the exact site of origin of which in the bronchial mucosa may be unidentifiable. Their microphotographs are quite compatible with anaplastic carcinoma of the lung, and the presence of a growth within the bronchi depicted in one of their figures is also significant. Indeed, the good description of their case given by these authors permits a fairly confident diagnosis of pulmonary carcinoma.

Geschickter and Copeland (1936)[9] tabulate 125 cases of "Ewing's sarcoma," only 12 of which came to autopsy and no details of which are given. These writers admit the possible clinical and histological resemblance between Ewing's tumour and metastatic neuroblastoma in bone, but of the latter they state: "The lesions, however, are radioresistant, which differentiates this disease from Ewing's sarcoma." This is the exact reverse of the opinions of Connor and of Stewart cited above, and is also refuted by the radiotherapeutic results in the case reported by Colville and myself and in the present case. Of "Ewing's sarcoma" Geschickter and Copeland also state: "Against the opinion, occasionally voiced, that this lesion is a metastatic tumor arising primarily outside of bone, is the failure to demonstrate such a primary focus in any of the cases studied and the occasional cures by amputation." The first of these two arguments, even if correct, is surely poor logic. In view of the clearly inadequate nature of most of the reports of "Ewing's sarcoma" and of the uncritical nature of the discussion regarding the identity of the growths in most cases with autopsy records, "failure to demonstrate" possible primary growths outside bone is not surprising. But Geschickter and Copeland are in error in speaking of "failure to demonstrate such a primary focus *in any of the cases studied*" (the italics are mine); they omit reference to the case described by Colville and me in 1933 and to my review of the subject in 1934, and they ignore Connor's admission cited above that some of the cases originally described by him as "Ewing's tumor" were undoubtedly metastatic. Geschickter and Copeland's second argument for the

primary nature of "Ewing's sarcoma," namely that "occasional cures by amputation" are obtained, is also very unconvincing since they themselves, like many other writers, stress the frequent difficulty of clinically distinguishing this disease from chronic osteomyelitis and from syphilis of bone, both of which have undoubtedly been responsible for many diagnostic errors in the past, and since, as Connor's paper cited above shows, confusion with osteogenic sarcoma is also possible.

Campbell and Hamilton (1938)[21] give an adequate autopsy report of a case of a radiosensitive tumour of the humerus in a girl aged 20 years, which they diagnose as a "Ewing's tumor." The autopsy revealed a large retroperitoneal growth internal to the left sacro-iliac joint with erosion of the bone, extensive neoplastic disease of the pancreas, and almost total destruction of the left adrenal gland by tumour. Although the authors admit that the possible primary nature of the adrenal tumour cannot be excluded, they say: "It is almost inconceivable to us that the tumor in the left suprarenal gland could have been a primary focus in this patient for such a long time, and yet have remained so small. . . . The long duration of symptoms, which is so often the case before any definite diagnosis can be made, argues against Ewing's tumor as having its primary origin in some organ apart from the skeletal system." These views, however, will fail to impress experienced pathologists, who are well aware of the frequent development of bulky, clinically obtrusive metastases from small, unprogressive and symptomless primary growths (see many examples recorded by Willis, 1933,[22] and by Trinca and Willis, 1936 [23]). Moreover, the authors apparently accept the case reported by Colville and myself as one of neuroblastoma, although the primary adrenal tumour was a very small one. Lastly, Campbell and Hamilton,[21] in discussing their case, ignore the possibilities that either the pancreatic or the pelvic growths may have been primary. The pelvic growth particularly, lying internal to the sacro-iliac joint, may easily have been, like the tumour in the present case, a neuroblastoma of the left sympathetic chain.

Under the title "Primary endotheliomyeloma (Ewing's tumor) of the sacrum," Brav and Rechtman (1938)[24] report a tumour in a child of 10 years, with autopsy. The large sacral growth destroyed bone in the vicinity of the right sacro-iliac joint and also

involved the lower lumbar vertebrae, and there was also a large retroperitoneal mass about 12 cm. in diameter close to the left kidney. The authors do not even mention the obvious possibility that either of these large tumours may have been neuroblastomatous.

A recent review of the classification of bone tumours (1939) by Ewing himself [25] indicates the widely divergent opinions that are still held regarding the nature of "Ewing' tumour," even by those who believe it to be a primary bone tumour. Geschickter and Copeland (whom Ewing strongly criticizes) regard it as a "lymphoma," while Oberling and Raileanu regard it as a totipotent myeloma of reticuloendothelial origin and think that the tumour cells have the capacity to form plasma cells, myelocytes, lymphocytes and even erythroblasts. Ewing disagrees with these views and insists that the tumour arises from capillary endothelium and is related to angiomas. He speaks of "pseudorosettes" and "characteristic perithelial structures" as being important structural features, and in the relationship of the tumour cells to blood vessels he sees evidence of "the angioblastic properties of the cells." In my opinion, Ewing's capillary hypothesis, like the views of Geschickter and Copeland, and Oberling and Raileanu, is purely speculative and has no basis in pathological experience.

Finally, turning from the writings of those who believe "Ewing's tumour" to be a primary growth of bone and a pathological entity, it is refreshing to read a paper written by Carl Sternberg (1935)[26] just before his death, in which he voiced a strong criticism of "Ewing's tumour." He described examples of metastatic growths which produced the Ewing syndrome, and he concluded that not only is the view that this syndrome denotes a pathological entity wholly unjustified, but also that the boundaries of the syndrome itself are quite uncertain. The foregoing review of some of the recent writings in this field fully endorses Sternberg's conclusions and justifies his thorough scepticism. Both clinically and pathologically the subject of "Ewing's tumour" will remain an unscientific chaos until an adequate number of cases presenting the usually accepted syndrome have been subjected to thorough autopsy study and the results have been adequately recorded and critically evaluated. To that end the present case has been described in some detail, and I believe it to be very significant that

it, like the only previous case with the Ewing syndrome seen at this hospital, should have proved to be one of metastatic neuroblastoma. I feel confident that when adequate study of a number of cases has been carried out, various types of metastatic growths, especially neuroblastoma, will be found to be most often responsible for the so-called "Ewing tumour." Further, although our present knowledge is insufficient to permit us to deny the possible existence of a primary bone tumour capable of giving the syndrome, it will not be surprising if future research should prove that no such primary tumour occurs.

Summary and Conclusions

1. A case of an adolescent girl in whom a large tumour of the femur was found, with characteristics conforming in every way with those of "Ewing's sarcoma" as usually described, and of over 3 years' duration, is reported. At autopsy the tumour was found to be a metastasis from a primary neuroblastoma of the left lumbar sympathetic chain.

2. Some recent publications regarding "Ewing's tumour" are reviewed, with the conclusion that the subject is chaotic, that the occurrence of a primary growth of bone of this nature is still unproved, and that metastatic growths of various types, especially neuroblastoma, will probably prove to be responsible for many of the cases.

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DESCRIPTION OF PLATE

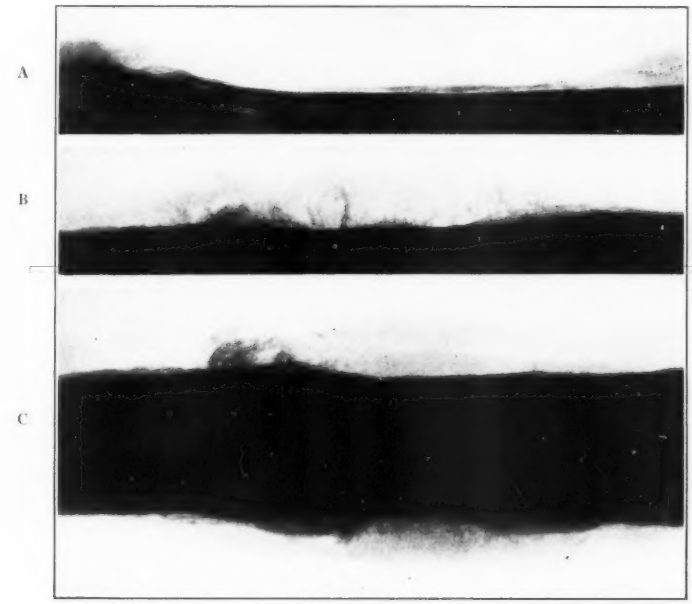
PLATE 67

FIG. 1. Positive prints from three parts of the skiagrams of the femur after removal: (*a*) shows "onion-skin" layers on the upper part of the shaft; (*b*) shows vertical bone spicules in the growth near the middle of the shaft viewed anteroposteriorly; and (*c*) is a side-to-side view of the middle of the shaft showing both vertical spicules and "onion-skin" layers of new bone in the growth.

FIG. 2. Diagram showing distribution of the retroperitoneal tumour "T," the femoral growth, and associated lymph glandular metastases, as seen after removal of the viscera and peritoneal deposits of growth.

FIG. 3. Microphotograph of a section of the retroperitoneal growth showing distinct rosettes in the otherwise diffuse round celled tumour tissue. × 120.

FIG. 4. Enlarged view of part of Figure 3 showing the rosettes, each containing a tangle of fine fibrils. × 350.



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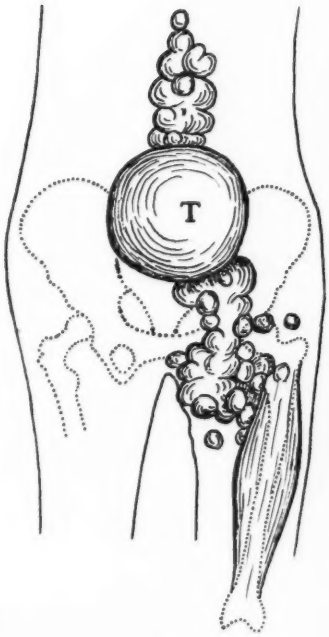

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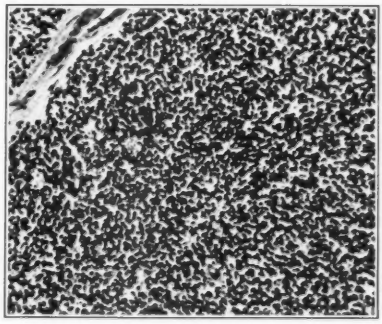

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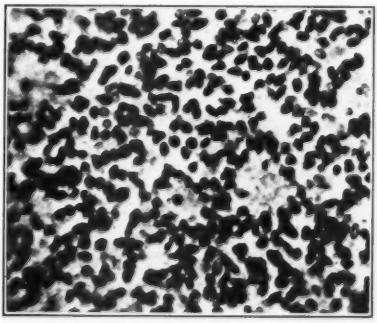

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Willis

Metastatic Neuroblastoma in Bone

UNIV. OF MICH.

INFECTIOUS FELINE AGRANULOCYTOSIS *

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Infectious feline agranulocytosis is a disease of the domestic cat characterized by leukopenia and pronounced neutropenia. The condition was first described by Lawrence and Syverton in 1938,[1] when it was reported that the evidence suggested a filterable virus as the etiological agent. Since then the viral etiology has been satisfactorily established as a part of an extensive study of the disease which is being carried out at the University of Rochester. The object of the present communication is to present in detail the hematological findings, together with a brief description of the clinical and pathological aspects of the disorder. The clinical features have been described previously.[1] Since an adequate description of the pathological picture of the identical disease has been made by Hammon and Enders,[2] our description of the pathology, which is for the most part confirmatory, is brief. A detailed presentation will be made later of the bacteriological observations, which include the viral studies, methods of transmission and host range.

The data which are used in this report have been collected from observations made on 113 animals suffering from this disease.

CLINICAL COURSE

The clinical course is in all essentials the same as that reported in our previous communication. A typical animal appears well for 5 or 6 days after exposure to the infectious agent. We have been unable to elicit any tell-tale symptom during this period of incubation. Following the incubation period the stage of clinical disease is gradual in its onset and variable in its manifestations. Infected cats may appear entirely well, or occasionally suffer a

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slight impairment of appetite. Other animals become listless, assume a recumbent position and refuse all food, usually from the 6th to the 9th day. Vomiting, diarrhea, and nasal and ocular discharges may occur irregularly. Death may intervene at any time after the animal refuses food. In some instances, in fact in many, this may occur before listlessness and weakness develop. It is not at all uncommon to leave an animal in apparent good condition and find it dead upon returning a few hours later. Oral or perianal lesions have never been noted. Enlargement of the peripheral lymph nodes has not occurred. If recovery is to take place, it usually begins after 2 to 4 days of the disease. The first clinical evidence for this is ingestion of food by the cat. Once the animal begins to eat, one can be fairly sure of eventual recovery. The period of recovery usually requires only 5 or 6 days.

Pyrexia: Fever is not a prominent symptom. Sixteen animals were carefully observed with reference to pyrexia. To establish the limits of temperature variability within this group of 16 cats, the rectal temperature for each cat was recorded on 10 of the 18 days that constituted the period of isolation, or control period. Based on these 160 readings, the mean temperature was 38.5° C. Following inoculation, temperature readings were made daily. Of these 16 animals, 6 had fever (a temperature above 39.4° C. was arbitrarily accepted as indicating fever) 1 day before the height of the disease and 10 had no fever at this time. Observations were made on only 12 of these animals at the height of the disease; 6 were found to have fever, and 6 to have no fever.

Weight: The disease is too acute and too short in duration for much loss of weight to occur. Of 11 cats with the disease, 5 showed no loss of weight, 4 showed a loss of 114 to 228 gm., and 2, a loss of only 57 gm.

Mortality: The experiments which have been conducted justify only a tentative statement as to the percentage of animals that succumb to the disease. We do know, from the number of animals that have died, that the mortality is quite high. Thus, of 110 animals with the disease, 51 died, 33 were killed * and 26 recovered. If one assumes that the mortality rate would have been the same for the 33 sacrificed animals as for the others, 22 of these animals

* Throughout these experiments ether was used as the lethal agent and for all operative procedures.

would have died. Therefore, 73 out of 110 cats with the disease would have succumbed, giving a mortality rate of 66 per cent.

The Blood

The White Blood Cell Picture

Detailed white blood cell studies were made on 83 cats with the disease. In addition, a sufficient number of white blood cell counts were made on the other 30 cats to establish the presence of leuko-

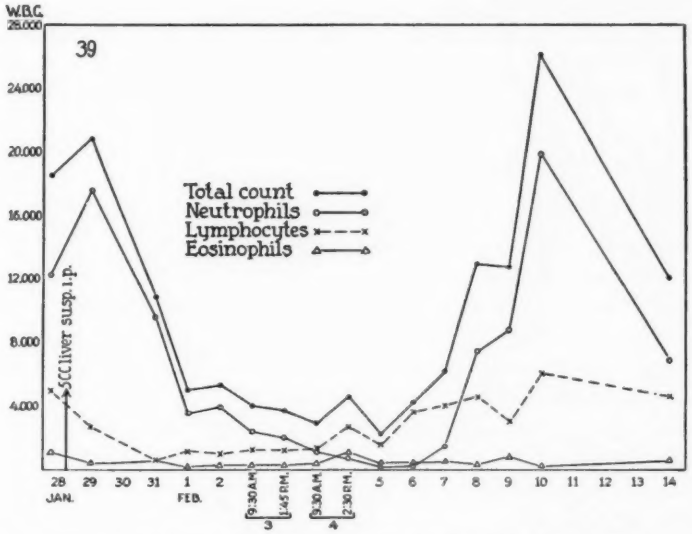

Chart 1. White blood cell findings in a cat of the "gradually declining type."

penia and neutropenia. Sixty-three of the 83 cats studied had only one or a few counts made prior to known exposure to the infectious agent. Counts were made on these animals repeatedly thereafter until death occurred or until recovery was under way.

The additional 20 cats were studied as follows: 18 had white blood cell counts and differential counts at 1 to 3-day intervals for 18 or 19 days prior to exposure; the other 2 had the same observations made at 2 or 3-day intervals (except for a single 5-day period) for 48 days prior to exposure. Following exposure, 16 of these cats had daily white blood cell studies until death occurred,

until they were killed at the height of the disease, or until they returned to normal. The remaining 4 animals of this group of 20 had essentially the same studies following exposure to the infecting organism, the only difference being that blood studies were omitted for a few days.

The animals all fell into two groups on the basis of the type of response shown by the white blood cells. We have designated these

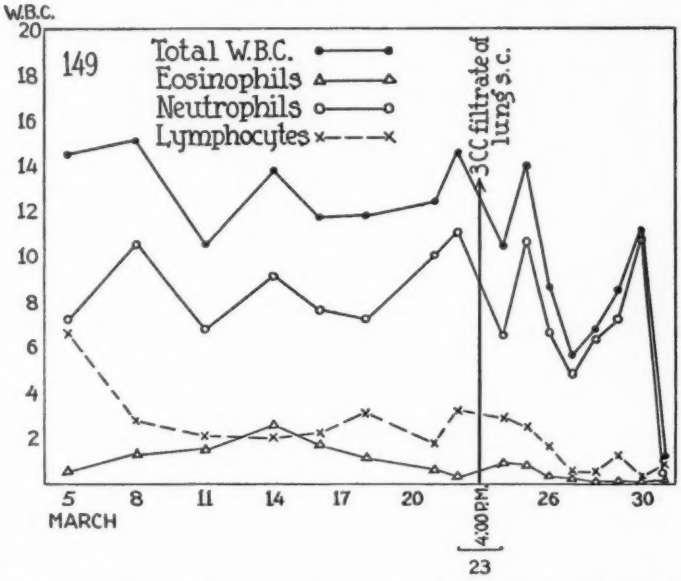

CHART 2. White blood cell findings in a cat of the "precipitously declining type."

groups as the "gradually declining" and the "precipitously declining." In the former group the total number of the white blood cells and the absolute number of the neutrophils gradually diminish from a few days after exposure to the infectious agent until the height of the disease. A typical response of this sort is shown in Chart 1. In the latter group, the precipitously declining, the total white blood cell count may diminish but little, if any, for the first 5, 6 or 7 days. The percentage of neutrophils rises, however, so that the absolute number of neutrophils approaches more and more closely the total number of the white blood cells. When

the point is reached where the neutrophils constitute almost all of the white blood cells (on the 5th to the 7th day usually), there is a sudden precipitous drop in the total number of the white blood cells, the number going to zero in some instances. A typical response of this sort is shown in Chart 2. Of the 83 animals used in this analysis, 42 had the gradual curve and 41 the precipitous. The mortality was much higher in the latter group.

Total Number of White Blood Cells: The average total white blood cell count for 113 cats at the height of the disease was 1350 per cmm., as compared with our normal of 15,000 per cmm. (average of 826 counts in 100 animals). Of the 113 animals, 22 had total white blood cell counts from 0 to 200 per cmm., whereas 19 animals had counts above 3000 per cmm. A total count as high as 6400 was found in only a single animal.

Differential Count

Neutrophils: The most marked abnormality in the blood picture was found in the differential count. The neutrophils were more markedly affected than any of the other white blood cells. At one time during the course of the disease 80 of the 113 animals used in this report had from 0 to 200 neutrophils in their peripheral blood. At the height of the disease 30 of these 80 had no neutrophils in their peripheral blood; 37 had less than 11 per cent, and only 6 had more than 60 per cent. Of the latter 6, all had a definite leukopenia, 1 having only 500 cells per cmm. The distribution of animals with reference to the absolute number of neutrophils is shown in Chart 3, while Chart 4 gives the findings with reference to the percentage of neutrophils at the height of the disease. These data show clearly the marked effect which the infection has on the neutrophils.

Lymphocytes: The total number of the lymphocytes was generally diminished at the height of the disease, as one would expect with such a pronounced leukopenia. In the majority of instances, however, there was a relative increase in the lymphocytes, the percentage being high in the differential count. Thus, 90 of 111 animals had more than 50 per cent of lymphocytes in the peripheral blood at the height of the disease, while only 5 of these cats had above 3000 lymphocytes per cmm. These cells appeared normal in every respect. While the relative percentage of lymphocytes

was generally increased at the height of the disease, it was usual in the precipitously declining group of animals to have both the percental and the absolute number of these cells fall just prior to the height of the disease. Furthermore, in both groups of animals, *i.e.* the gradually declining and precipitously declining, an absolute lymphopenia was demonstrable during the greater part of the incubation period. (This observation is in agreement with the re-

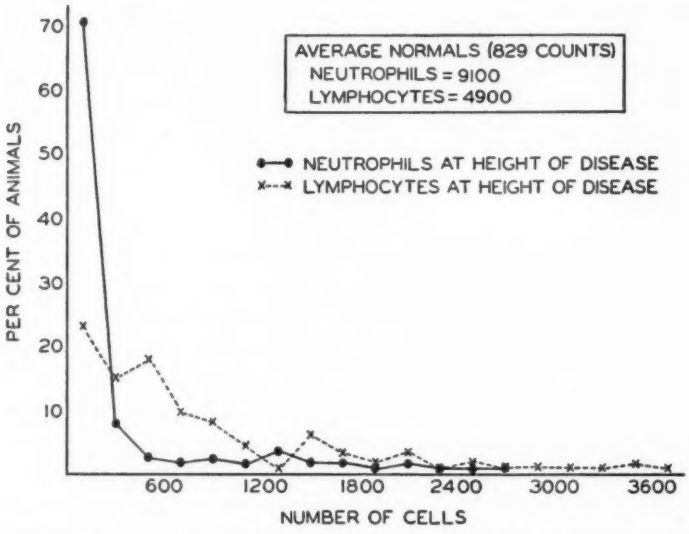

CHART 3. Curves showing the distribution of animals with regard to the number of neutrophils and lymphocytes.

sults Plum [3] has reported for human agranulocytosis secondary to the ingestion of amidopyrine.) In a few instances a definite lymphocytosis was observed during the period of recovery. In Charts 3 and 4 the distribution of the animals with reference to the total number and the per cent of lymphocytes at the height of the disease is plotted.

Eosinophils, Basophils and Monocytes: No consistent changes were noted with reference to these cells. The impression was gained at times that the eosinophils were increased at the height of the disease, but analysis did not bear this out. The average percentage of eosinophils in 20 animals at the height of the dis-

ease was 13 per cent, as compared to an average figure of 9 for the same animals prior to inoculation. In isolated instances the percentage of eosinophils was quite high (as much as 80 per cent).

"Stab" Cells: The "stab" cell proved to be an important part of the white blood cell picture in these animals. Normal cats only rarely showed "stab" forms in the peripheral blood. In the typi-

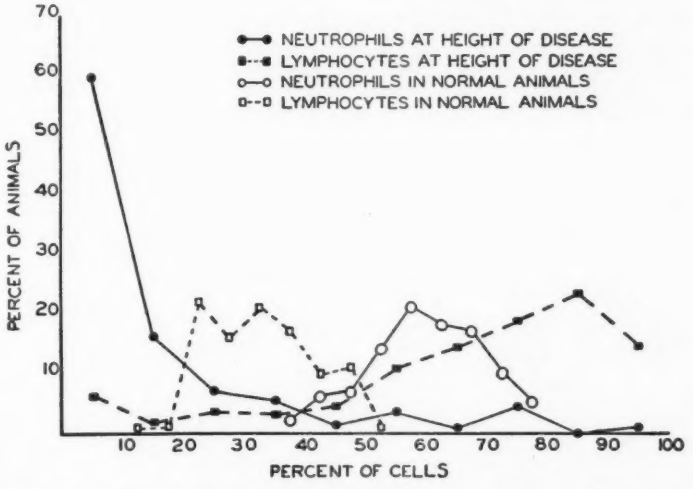

CHART 4. Curves showing the distribution of animals with regard to the percentage of neutrophils and lymphocytes. For comparison, curves are shown for values obtained for these cells in counts on 100 normal animals.

cal animal with the disease an increased number of "stabs" occurred on the day before the height of the disease or at the height. The increase was usually slight in amount. With recovery, however, a pronounced increase in the number of these cells occurred. In some instances there were over 30 per cent of "stab" forms. This marked "shift to the left" persisted for 4 or 5 days.

The Red Blood Cell Picture

Determinations of the total number of red blood cells in the peripheral blood of 21 cats were made prior to the active stage of the disease and during its height. Fifty-nine red blood cell counts

were made prior to the height of the disease and 23 counts during the height of the disease. The average figure for the total number of red blood cells before the height of the disease was 7,140,000 per cmm., as compared with 6,880,000 per cmm. at its height. On 9 of the 21 cats repeated red blood cell counts and hemoglobin determinations were made prior to the height of the disease in order to reveal any changes which might otherwise be masked by the general average. Identical results were obtained. An occasional animal showed lower counts at the height of the disease than in the pre-exposure period. On the same 9 animals observations were begun at the following time intervals before the height of the disease: 10 days (1 animal); 11 days (2); 13 days (2); 14 days (1); 15 days (2); and 32 days (1). The lowest number of determinations prior to the height of the disease was 3 and the greatest was 23, the average being between 7 and 8. These observations were continued throughout the height of the disease and during recovery. We have satisfactory studies on only 3 animals during the period of recovery. Two of these animals were studied until the 8th day and 1 until the 11th day following the height of the disease. A slight tendency for the hemoglobin and red blood cell values to drop at the height of the disease or during the early days of recovery was observed, but the change was not great enough to be of significance. Slightly more nucleated red blood cells were noted in the peripheral blood of cats with the disease (particularly in the recovery period) than in normal cats. Otherwise, there were no changes in the red blood cell picture. In summary, slight evidence of anemia was found in a study of the peripheral blood.

Pathological Examination

Autopsies were performed on 45 animals. In the majority of instances the animals were killed when at the height of the disease, or else were autopsied a very short while after death. Grossly the organs showed little that was abnormal. In the lungs of some of the animals small hemorrhagic areas were apparent, but this was an inconstant finding. The small intestine, from about 12 inches below the pylorus to the ileocecal valve, was sometimes moderately injected. There were no other abnormalities to be seen in the gross.

Sections were obtained from 41 of the autopsied diseased animals in addition to those obtained from normal animals for purposes of control. The following tissues were prepared for histological study: heart, lung, liver, gall-bladder, bile ducts, spleen, kidney, kidney pelvis, ureter, urethra, urinary bladder, adrenal, pancreas, lymph node, esophagus, stomach, duodenum, ileum, jejunum, colon, bone marrow (humerus, femur, vertebrae and ribs), nervous tissue (including various areas in brain and cord), parotid gland, submaxillary gland, testicle, epididymis and tonsil. The histological changes characteristic of their infection with the virus were: necrobiosis, as evidenced by cells in various stages of degeneration; proliferation, as shown by the extensive increase in the reticular cells, and by hypertrophy of inclusion-bearing cells; and the formation of intranuclear inclusion bodies.

The most significant feature of the histopathological picture was the presence of intranuclear inclusion bodies (Cowdry's type A). These inclusion bodies were found only in sites where one could attribute the pathological alterations to the specific infection. They were typically acidophilic and were variable in size, shape and structure. In their internal structure they ranged from compact, rather homogeneous and well organized bodies to loosely dispersed, discrete granules. In nuclei with inclusion bodies there was an accompanying margination to the nuclear membranes of both the nucleolus and the chromatin network, resulting in a clear space or "halo" around the inclusion body. The inclusion-bearing cells varied in size from normal to greatly hypertrophied and vesiculated cells. Inclusion bodies were found only in the epithelial cells of the gastro-intestinal mucosa, in reticular cells of the lymphoid tissue (intestine, lymph nodes, spleen and tonsils), and in the alveolar epithelium of the bronchial mucous glands.

Significant histopathological changes were limited to the hematopoietic system and to the gastro-intestinal tract. The remaining tissues were essentially normal. It was noted that the neutrophils were absent or markedly diminished in the lungs and the liver.

Spleen

Thirteen sections from 13 animals were examined. The capsule was intact. The follicles were less prominent than in normal animals. There was considerable proliferation of the reticuloendo-

thelial tissue throughout the organ. In most instances the reticular cells in the germinal centers were increased in number. At times this picture was markedly abnormal, and at others there were only slight alterations. The stroma appeared normal. There were no neutrophils and no megakaryocytes. Furthermore, there was no phagocytosis of red blood cells or pigment except in a single instance where it was slight. In a few sections extensive hemorrhage was present, but in most of them hemorrhage was conspicuously absent. A microphotograph of the spleen at a low magnification (× 100), showing typical changes, is reproduced in Figure 14.

Lymph Nodes

Forty-five sections from 16 animals were examined. The general architecture was retained. The capsule was intact. The most striking change was the marked hyperplasia of the reticuloendothelial cells. This was particularly prominent in the medullary portion of the node. Many of the germinal centers appeared to be replaced by reticuloendothelial cells, but, in each node, considerable numbers of immature lymphoid cells were found. Lymphocytes were most numerous about the cortical portions of the lymph nodes where they did not appear to be appreciably decreased in numbers. An occasional observation was the presence of neutrophils among the hyperplastic reticulum in the central portions of the node. In a few of the specimens there was marked phagocytosis of hemoglobin by the reticuloendothelial cells. The changes that occur in lymph nodes are shown in Figure 13.

Gastro-Intestinal Tract

Seventy-one sections, taken at several levels from each division of the gastro-intestinal tract, were obtained from 10 cats. With the exception of sections removed from the esophagus, every section yielded evidence for a specific viral infection, as shown by the presence of Type A (Cowdry) intranuclear inclusion bodies in the epithelial cells of the mucosa (Fig. 18). The distribution of inclusion bodies was highly variable, ranging from one or two in an entire histopathological section to a score within a single high power field. The further histopathological changes, which were largely confined to the lateral and basilar portions of the

villi, varied from hypertrophy of the individual cells with questionable circumferential mononuclear invasion to complete necrosis and, in certain instances, sloughing of both villous and acinar epithelium. In no instance was the involvement of the gastro-intestinal tract extensive or of a grossly ulcerative nature. On the contrary, the lesions were characteristically minimal in extent and were limited to the mucosa and lymphoid tissue. Irrespective of the extent of the involvement, the architectural pattern characteristic of the gastro-intestinal mucosa was maintained. The changes in the mucosal epithelium varied from animal to animal and even in the same animal. On study, one of four pictures, or any combination of them, might be presented. Epithelial cells that were: (1) *normal*, except for a very occasional cell with an intranuclear inclusion body; (2) *flattened and pyknotic*, either individually or in groups; (3) *necrotized*, both *in situ* and lying in the glandular lumens; and (4) *sloughed*, leaving the denuded basilar membrane behind. The cellular invasion that accompanied the epithelial derangement consisted mainly of mononuclear cells. Occasional clumps of polymorphonuclear cells were present, however, and these cells persisted in the necrotic portions, even though the peripheral blood had been entirely depleted of polymorphonuclear cells.

Esophagus: No histopathological changes were present.

Stomach: The only apparent abnormality in the fundus was limited to an occasional intranuclear inclusion body. In the pylorus, however, the damage to the epithelium was quite extensive with denudation and flattening of the cells.

Small Intestine: Although scattered lesions were present throughout, the involvement of the mucosa appeared to be more consistent and extensive toward the iliac portion of the intestine. In every animal examined, the sections from the ileum showed more extensive involvement than any other region of the intestine.

Colon: The colon showed much less involvement than the small intestine. When microscopic changes were encountered they were largely confined to the proximal portion of the colon with only slight evidence for the infectious process in the distal portions. In certain instances the histopathology in the proximal portion of the colon closely simulated that of the lower ileum.

For control purposes 17 sections from 5 normal cats were

carefully studied. In none were inclusion bodies or other changes characteristic for the disease found.

Central Nervous System

No abnormalities were noted on gross examination of the central nervous system, either ***in situ*** or after removal from the body, in the group of 7 animals killed at the height of the disease, or in the group of 3 normal animals which were killed for control purposes. The brains and cords of all of the animals were studied. In the diseased group microscopic study of 134 sections, representing every division of the central nervous system, failed to reveal any abnormality that could be ascribed to infection with the virus of infectious feline agranulocytosis. No cytoplasmic or intranuclear (either Type A or B) inclusion bodies were present. A similar study of 40 sections from the 3 normal animals also failed to reveal any abnormalities.

The Bone Marrow

The bone marrow was studied in detail, for the clinical picture pointed to this organ as the principal point of attack by the infectious agent. On gross examination the marrow appeared to be fatty and aplastic, or normal. (We agree with Custer [4] that little significance can be attached to the gross appearance of the marrow.)

For histopathological study specimens were fixed in Zenker's fluid (plus 5 per cent acetic acid), decalcified and embedded in paraffin for sectioning. The sections were stained with hematoxylin and eosin. Our studies may be divided as follows:

Group I: Observations on the bone marrow of animals killed at the height of the disease.

Group II: Observations on bone marrow biopsied from individual animals at different stages of the disease.

Group III: Observations on the bone marrow of animals killed at various periods during recovery.

Group IV: Differential counts on smears made from suspensions of bone marrow removed from animals at various stages of the disease.

Group I: In the 1st group, bone marrow was studied from 17 cats killed at the height of the disease. From 14 of the 17 animals,

marrow was obtained from the following bones: vertebra, rib, femur and humerus. Each of the animals yielded bone marrow with essentially the same cellular picture. The myeloid series of cells was most markedly affected. The almost complete lack of differentiation was characteristic and resulted in one predominant type of myeloid cell, probably the myeloblast type. These cells had much the same characteristics as those described by Custer.[4] The nuclei were large, vesicular, and lacking in specific granulation. Usually they contained one or more large nucleoli. The number of mitotic figures was not abnormal. There were no juvenile, stab or segmented forms in most of the specimens. The erythroid elements persisted more than the myeloid and in some animals were abundant. The megakaryocytes were undiminished in number and normal in appearance. Even with extensive hypoplasia, there was a definite sprinkling of megakaryocytes. Extensive congestion was evident in all of the bone marrow sections. Eosinophils were present in smaller or larger numbers. In a single animal the majority of the cells in the vertebral marrow were eosinophils. In a specimen from another animal a lymphoid follicle was present. The histopathological changes that typify the reaction of the bone marrow in cats at the height of the disease are illustrated in Figures 9–12. In these microphotographs it may be seen that the characteristic type of cellular reaction persists even when there are extensive quantitative differences in the total number of cells present.

Group II: Serial biopsies of the femoral or humeral marrow were performed on 6 cats at various stages of the disease. Marrow was obtained on three occasions from 5 of the cats and only twice from the remaining animal. The day of the biopsy in relation to the height of the disease and the findings are recorded below.

Two days before height of disease. A single specimen revealed many typically undifferentiated cells, a fair number of later myeloid cells and scattered erythroid cells. The cellular picture was similar to that for marrow in the stage of recovery.

One day before height of disease. The salient observation in two specimens was the absence of practically all late myeloid forms. The myeloid series was represented almost entirely by undifferentiated cells. There were many erythroid cells. Mod-

erate numbers of megakaryocytes and eosinophils were seen. There were a few mitotic figures.

Height of disease. The picture was identical with that given for the autopsied animals, *i.e.* most of the cells present were undifferentiated cells of the myeloid series and erythroid cells.

1st day of recovery. Two specimens showed a tremendous increase in the number of cells in the marrow as compared with the number at the height of the disease. The great majority were undifferentiated myeloid cells but there was some beginning differentiation into stab forms. Mitotic figures were noted frequently. There were few erythroid cells. Few to moderate numbers of megakaryocytes were seen.

2nd day of recovery. Three specimens were studied. The cells were present in normal or increased numbers. The differentiation of the myeloid cells had advanced so that more of the late forms were seen. Mitotic figures were frequent (up to three per oil immersion field). Erythroid cells appeared normal, as did megakaryocytes. A few eosinophils were present.

4th day of recovery. Three specimens were studied. The striking part of the picture at this stage was the large number of cells. These showed well marked differentiation into late myeloid forms, but there were still a good many undifferentiated cells. Frequent mitotic figures (up to five per oil immersion field) were found. Normal numbers of erythroid cells and megakaryocytes were present.

Group III: In addition to the serial bone marrow biopsies, 6 animals in various periods of recovery were killed for specimens of marrow from the vertebra, rib, femur and humerus. The findings were similar to those recorded for the serial specimens. In Figures 1–8, microphotographs in both low and high magnifications, are the bone marrow of the normal cat (1 and 2); bone marrow of a cat at the height of the disease (3 and 4); bone marrow of the same animal on the 1st day of recovery (5 and 6); and bone marrow of this animal on the 4th day of recovery (7 and 8).

Group IV: For differential counts, specimens of marrow were obtained from the humerus or the femur 2 and 1 day before the height of the disease; at the height of the disease; and 1, 2, 3 and 4 days after the height of the disease. The marrow was macerated in human blood serum to yield suspensions for making smears on

TABLE I

Average Differential Marrow Counts (Per Cent)

	Normal	2 days before height	1 day before height	Height of disease	1st day of recovery	2nd day of recovery	3rd day of recovery	4th day of recovery
Number of counts	13	1	2	13	4	5	1	5
Eosinophils	1.5	0.8	2.9	4.2	0.6	2.0	0.0	0.8
Myeloblasts	1.3	1.8	2.4	1.8	3.3	2.9	3.0	2.0
Promyelocytes	7.6	3.2	4.8	2.5	8.9	5.9	8.8	6.2
Myelocytes	4.6	0.8	3.2	2.7	5.2	8.3	3.0	5.6
Juveniles	9.8	3.8	1.2	0.5	8.1	11.0	4.2	17.2
Stabs	13.4	17.6	2.0	0.6	7.8	13.9	11.2	20.1
Segments	5.3	6.8	0.5	0.2	0.6	1.6	3.4	4.1
Total myeloid cells	43.5	34.8	17.0	12.5	34.5	45.6	33.6	56.0
Lymphocytes	7.8	2.0	3.8	8.9	4.5	2.0	2.6	2.0
Plasma cells	0.3	0.4	0.3	0.17	0.2	0.2	0.0	0.1
Megaloblasts	0.7	1.2	0.7	0.9	0.7	0.24	0.0	0.5
Erythroblasts	6.3	15.0	12.8	6.1	3.4	3.8	5.0	7.4
Normoblasts	8.3	11.4	10.3	5.4	6.4	6.6	2.4	6.1
Total erythroid cells	15.3	27.6	23.8	12.4	10.5	10.6	7.4	14.0
Unclassified cells	6.2	9.6	7.8	3.4	4.9	2.5	4.8	2.2
Degenerated cells	26.3	25.6	47.3	62.6	45.8	39.3	51.6	25.6
Erythroid myeloid ratio	0.35	0.8	1.4	1.0	0.3	0.23	0.22	0.25

glass slides. After fixation the smears were stained with Wright's stain or occasionally with the oxydase stain. Of the 44 preparations that were stained with Wright's stain, 13 represented marrow from normal animals and 31 marrow from sick animals.

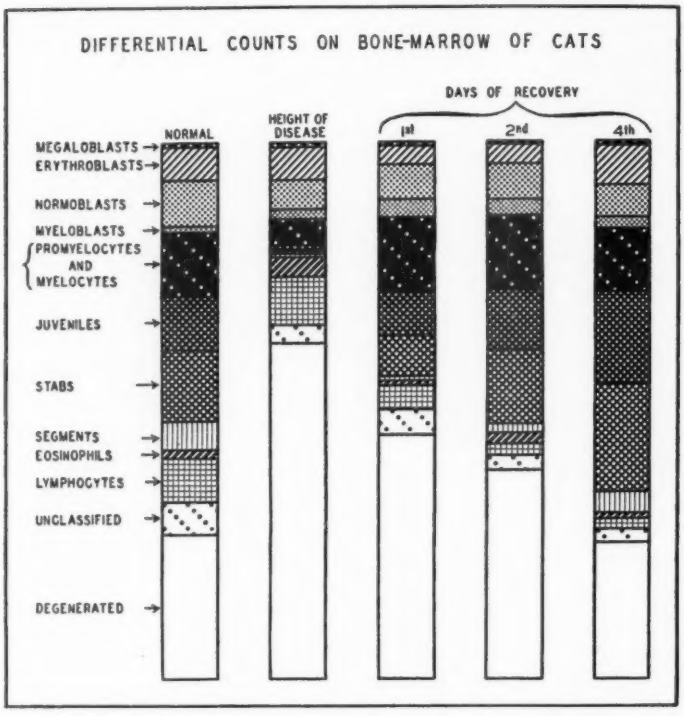

CHART 5. Graphic representation of differential bone marrow counts. The marked diminution at the height of the disease in the percentage of myeloid cells, particularly the juveniles, stabs and segments, is in striking contrast to almost normal percentages of erythroid cells. Note that there is a very rapid return of the myeloid cells during the recovery phase.

Differential counts were made of the nucleated cells in each of these preparations, 500 cells being counted in each instance. The results of these differential counts are shown in Table I and in Chart 5. In brief, the principal finding was a marked diminution in juveniles, stabs and segments at the height of the disease. The

eosinophils were slightly increased at this time. Following the height of the disease there was a pronounced increase in the number of late myeloid cells until on the 4th day they were present in greater than normal numbers. The erythroid cells showed no constant variation and usually were present in normal numbers at each stage of the disease. In summary, it may be said that the differential counts yielded results that agree with those obtained from a study of the fixed stained sections.

Discussion

The results of the present studies indicate that there are many points of similarity between infectious feline agranulocytosis and human agranulocytosis. The white blood cell picture in the feline disease simulates that seen in the human disease. In both conditions there are sudden drops in the total white blood cell count and in the number of neutrophils. These changes result in a relative increase in the number of lymphocytes. (See Chart 4 which illustrates the relative lymphocytosis.)* The marked "shift to the left," which occurred regularly in the feline disease during the phase of recovery, has been noted repeatedly in man. Moreover, the absence of thrombopenia and the inappreciable anemia parallel the human malady.

A satisfactory comparison of the white blood cell changes in the pre-agranulocytic stage of the human malady with those in the preclinical period of the feline disease cannot be made since most of the human cases have been studied mainly after the development of pronounced neutropenia. Stephens and Lawrence [5] have studied in this laboratory a woman with cyclical agranulocytosis and in her case there was no antecedent lymphopenia. However, this represented an unusual type of the disease and the white blood cell responses may have been different from those in the usual case of agranulocytosis. The best opportunity for comparison is afforded by patients in whom the disorder has been produced a second time by the readministration of amidopyrine. Plum [3] has made a detailed study of seven patients of this type. Qualitatively the changes reported by him are of the same type as we have noted during this period in the cat. There is this dif-

* This chart also serves to contradict Hammon and Enders' statement that there is no constant relative increase in the number of lymphocytes.

ference: whereas he found a marked rise in the lymphocytes at the time of the primary marked fall in granulocytes, we found the rise in the lymphocytes to be inconstant and further it did not occur until the recovery phase had developed.

The cytological picture of the bone marrow is also essentially similar to that which has been reported for human agranulocytosis. Thus, the erythroid cells are not affected in the human disease. Similarly, in the feline disease these cells are present in normal percentages. Noteworthy is the fact that foci of erythroid cells were readily detected. It is probable, however, that the absolute number of erythroid cells in the bone marrow is reduced when the bone marrow is markedly hypoplastic.

No systematic study in human agranulocytosis has been made of the changes in the bone marrow which develop during successive stages of the disease. It has been possible to make such observations in the feline infection. We believe that our findings may throw light on the widely divergent descriptions of bone marrow from cases of human agranulocytosis. A most remarkable and regular finding is the rapidity with which changes have been observed to occur in the bone marrow. Two days before the height of the disease some diminution is detectable in the differentiation of the myeloid cells. This progresses until at the height of the disease there is no differentiation of the myeloid cells, all being early forms. At this stage there is usually a hypoplasia, but, in some of the animals, the number of cells may not be diminished appreciably. On the 1st day of recovery differentiation of the myeloid cells can be detected and within 4 days there is marked differentiation with an abundance of neutrophils, stabs and juveniles. In the light of the rapidity with which these changes occur it is easy to understand why one report states that the bone marrow in human agranulocytosis is aplastic, and another that the bone marrow contains a normal number of cells. In fact, one wonders why there is even as much uniformity in the literature as there is, if such changes occur as rapidly in man as they have been observed in the cat. The observations of Rohr [6] and Plum [3] bear out this opinion. With a realization that such sudden rapid changes in cell content and cell types do occur, it becomes immediately apparent that the results of a single study of the bone marrow should not be accepted as diagnostically con-

clusive. The fact that only a small portion of marrow is removed from a single isolated area in the usual biopsy emphasizes this statement.

With the exception of the involvement of the gastro-intestinal tract in the feline disease, the other pathological changes resemble those reported for the human condition. As points of contrast, however, the absence of oral and perianal lesions in the feline infection may be mentioned. Furthermore, the duration of the acute phase in the cat is less than in the human. There is probably a marked difference in etiology as well. The feline disease is caused by a virus, whereas the most likely explanation for the human malady is that it is the result of a chemical intoxication. Generalized blood stream infections by secondary invaders may occur in both diseases, probably as the result of the lowered resistance of the host.

By the foregoing comparison of an experimental agranulocytosis of known etiology, as it occurs in the feline host, with the disease as it has been observed in humans, we do not presume, of course, to imply that the two are the same. On the contrary, we wish to emphasize that they are not. Nevertheless, the similarity of the clinical and pathological pictures in the two hosts suggests that widely different agents may instigate similar reactions. Thus, the agranulocytosis itself may be regarded as a symptom, like fever, purpura, and so on, which are produced by a variety of agents. To make clear our line of thought we may refer to an analogous situation — the frequency with which lead encephalitis, an intoxication, simulates a viral encephalitis, an infection. This is due to the fact that the elements of the central nervous system can respond to injury in only a limited number of ways. Thus, while amidopyrine, sulfanilamide and other chemical compounds hold the foremost place in any consideration of the etiology of the human disease, it is by no means impossible that the responsible factor may be any one of a variety of other agents, such as a yet unrecognized virus or filterable agent.

Moderate leukopenia is present in most viral infections. In so far as we know, hog cholera is the only viral disease with a blood picture that even resembles that of feline agranulocytosis. The peripheral blood picture in hog cholera was described by Lewis and Shope,[7] but they did not report the pathological changes in

the various organs. It is only possible, therefore, to compare the peripheral blood picture in the two infections.

What explanation is there for the leukopenia and profound neutropenia of the feline disease? The neutrophils are known to disappear from the peripheral blood stream extraordinarily rapidly. Within a few hours a normal percentage of neutrophils may be supplanted by complete absence of neutrophils. We have seen this happen repeatedly. Such rapid disappearance cannot be explained on the basis of bone marrow insufficiency alone, for if this were the sole mechanism at work there would be a gradual diminution in the number of neutrophils, the older ones going first and then the younger ones. While the exact life of the neutrophil is not known, it has been estimated as from 3 to 5 days.[8, 9] Accordingly, if bone marrow insufficiency were the responsible factor, one would expect the cells to take this long to disappear. Aside from the failure of regeneration in the bone marrow, it is suggested, therefore, that a toxic substance is produced which may destroy neutrophils in much the same way as antineutrophilic serum.[10] Furthermore, there is evidence which indicates that such direct destruction does occur in the human. In fact, Fitz-hugh [11] has suggested such a possibility. It would seem, therefore, that a combination of at least two mechanisms may be held responsible for the leukopenia and neutropenia. These are, first, hypoplasia and maturation arrest of the myeloid cells in the bone marrow; and, second, direct destruction of neutrophilic leukocytes in the peripheral circulation by an, as yet, hypothetical toxic agent.

It is interesting that the platelets remain in normal abundance. This is what the findings in the bone marrow would lead one to expect, since megakaryocytes were found wherever there were marrow cells. In the light of Howell and Donahue's [12] idea that large numbers of platelets are made in the lungs of cats, our findings are the more readily explained, for we discovered no abnormalities in the pulmonary tissues.

The absence of significant anemia may be explained in two ways. First, the remaining marrow cells were observed to include a normal percentage of red cell progenitors. Thus, appreciable numbers of these cells remain in practically every instance, even though the total numbers of erythroid cells may actually be diminished when there is a pronounced hypoplasia of the marrow.

Second, the duration of the illness is too short to result in marked anemia on the basis of a cessation in the manufacture of red blood cells. Certainly this is true if we can assume that the average period of survival for red blood cells of the cat is similar to that of dog and man, *i.e.*, from 100 to 120 days.[13, 14, 15]

Summary

The clinical, hematological and pathological aspects of a recently discovered viral infection of cats, which we have named infectious feline agranulocytosis, are described. The disease is characterized by marked leukopenia and neutropenia in the absence of thrombopenia and appreciable anemia.

The histopathological changes are directly attributable to viral activity, as evidenced by intranuclear inclusion bodies (Type A, Cowdry), necrobiosis and proliferation. The most pronounced abnormalities have been found in the hematopoietic tissues and in the intestinal mucosa.

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DESCRIPTION OF PLATES

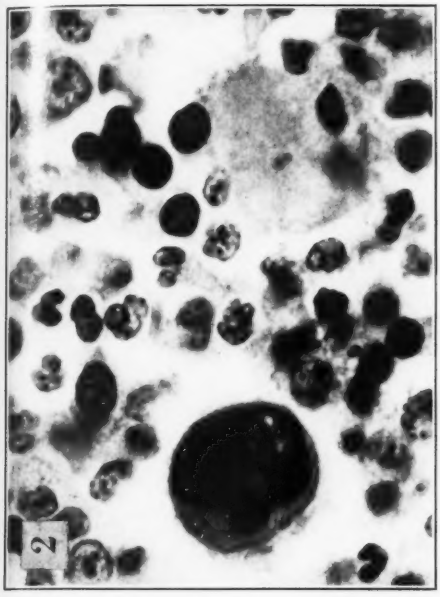
PLATE 68

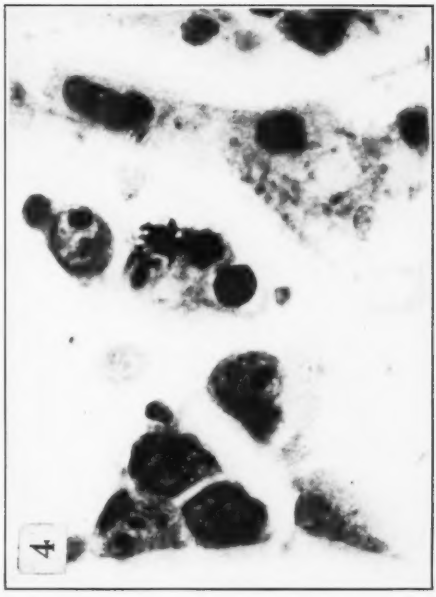
FIG. 1. Bone marrow from the humerus of a normal cat. × 100.

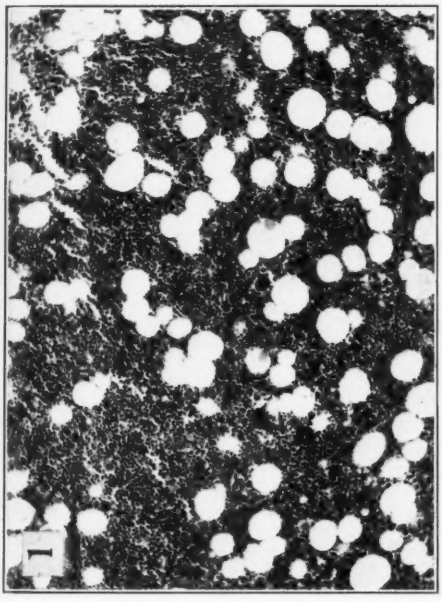
FIG. 2. High magnification of bone marrow from the humerus of the same normal cat. Note the wide variety of cells. × 1500.

FIG. 3. Femoral bone marrow of Cat 285 at the height of the disease. Note the marked hypoplasia of the specimen. × 100.

FIG. 4. High magnification of area from Figure 3. Note the undifferentiated cells with large vesicular nuclei. × 1500.







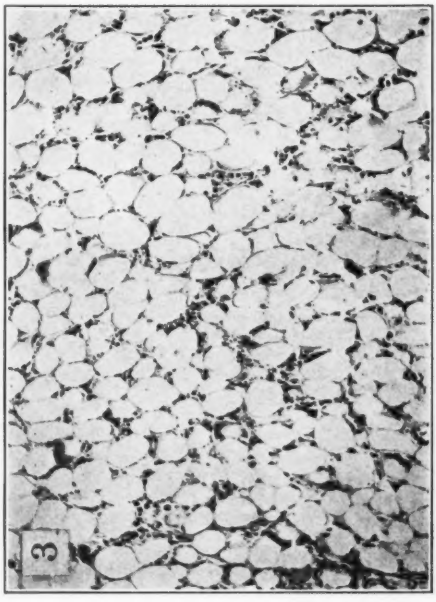


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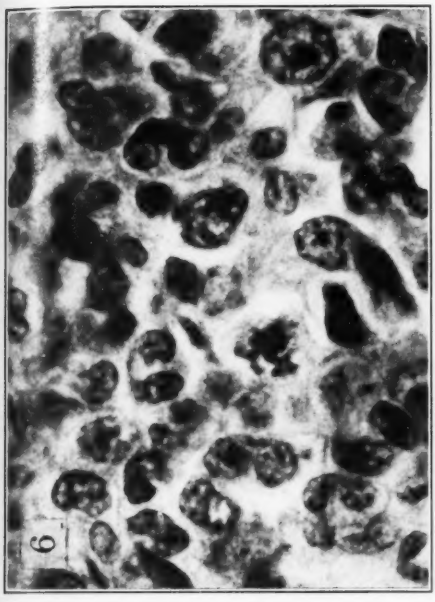
PLATE 69

FIG. 5. Femoral bone marrow of Cat 285 on the 1st day of recovery. Note the presence of distinctly more cells. × 100.

FIG. 6. High magnification of area from Figure 5. Many undifferentiated cells are still present but a few neutrophils can be seen. × 1500.

FIG. 7. Humeral bone marrow of Cat 285 on the 4th day of recovery. Note the presence of numerous cells — if anything, more than in the normal specimen. × 100.

FIG. 8. High magnification of an area from Figure 7. Undifferentiated cells are still present but the general cellular picture is approaching the normal. × 1500.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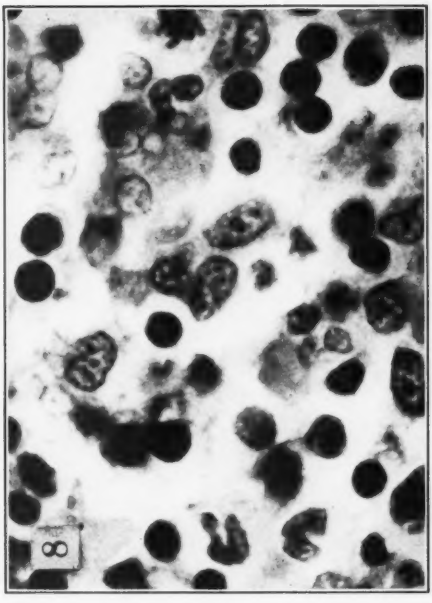
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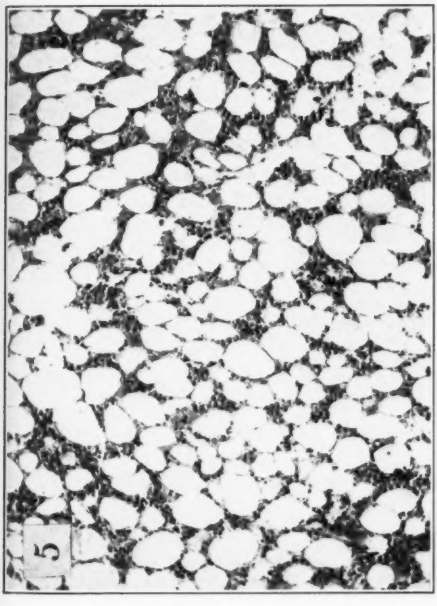
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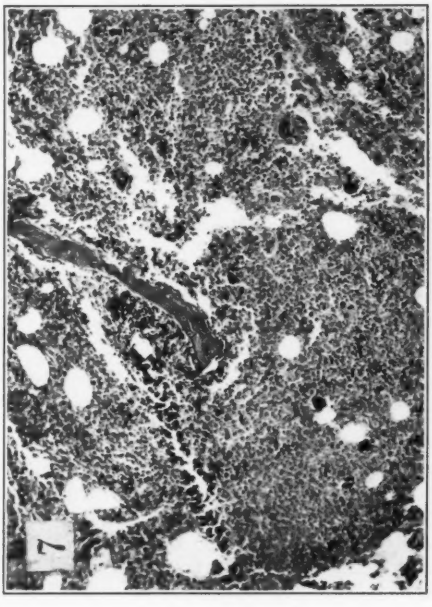
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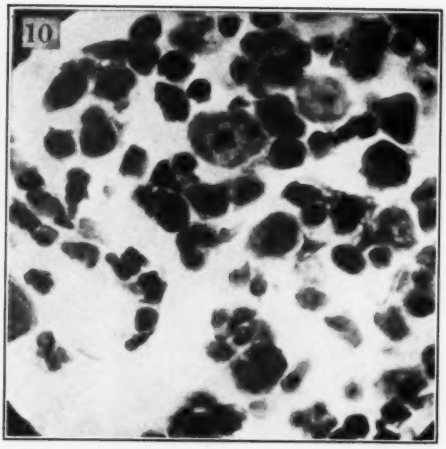
PLATE 70

FIGS. 9 and 10. Low (× 100) and high (× 1500) magnification of the vertebral bone marrow of Cat 77 at the height of the disease. Note the large number of cells present. The myeloid cells are for the most part undifferentiated. Many erythroid cells were found in this specimen.

FIGS. 11 and 12. Low (× 100) and high (× 1500) magnification of the vertebral bone marrow of Cat 55 at the height of the disease. This specimen shows marked myeloid hypoplasia. The qualitative changes in the cells are of the same character as those in Figures 9 and 10.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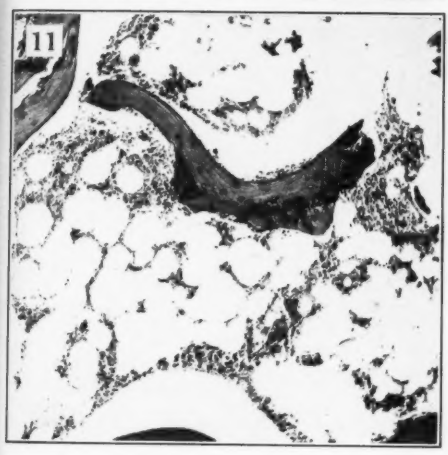
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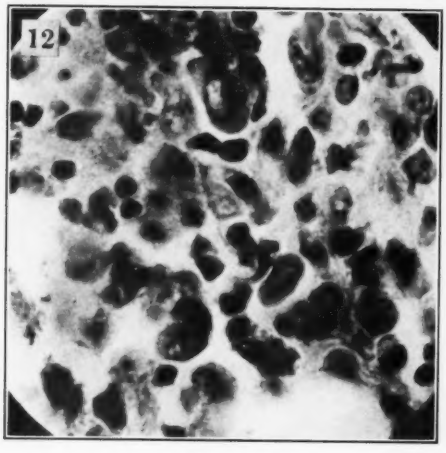
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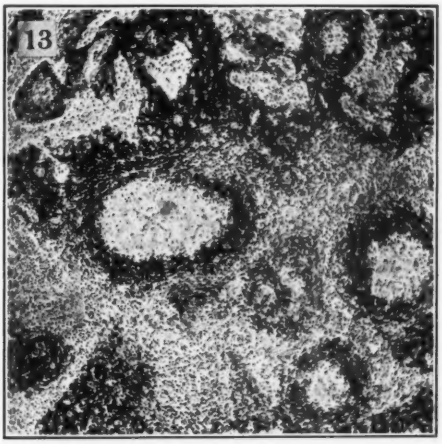
PLATE 71

FIG. 13. Lymph node from Cat 54 at the height of the disease. Note the marked proliferation of the reticuloendothelial tissue. × 100.

FIG. 14. Spleen from Cat 54. This shows marked proliferation of the reticuloendothelium also. × 100.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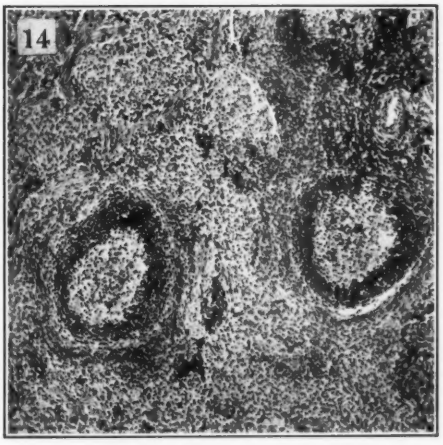
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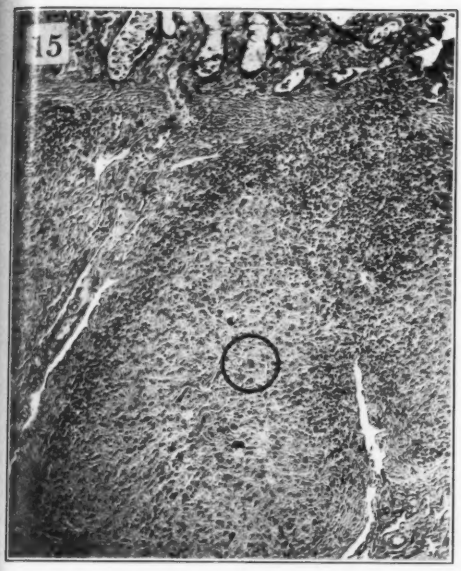
PLATE 72

FIG. 15. Low magnification of the wall of the terminal ileum of Cat 389. A large hyperplastic lymph node with its germinal center largely replaced by reticuloendothelial cells is shown. × 100.

FIG. 16. High magnification of the area within the circle in the germinal center of the lymph node shown in Figure 15. Four intranuclear inclusion bodies are shown, as indicated by arrows. × 1500.

FIG. 17. Low magnification of the terminal ileum of Cat 389. × 100.

FIG. 18. High magnification of the area within the circle in Figure 17. Three intranuclear inclusion bodies are shown, as indicated by arrows. × 970.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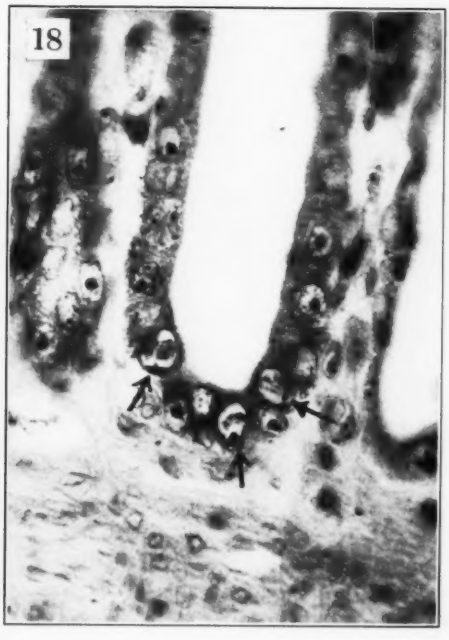
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PATHOLOGICAL CHANGES PRODUCED BY THE SUBCUTANEOUS INJECTION OF RATTLESNAKE (CROTALUS) VENOM INTO MACACA MULATTA MONKEYS *

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Accounts of the gross and microscopic lesions in fatal cases of rattlesnake (*Crotalus*) poisoning are rare. Willson [1] in 1908 collected 48 fatal cases, and of these there had been only 4 postmortem examinations recorded. Even in these cases the autopsies were incomplete and none included histological examination of the tissues removed.

Complete descriptions of the gross and histopathological changes in tissues following the administration of rattlesnake venom are based almost entirely on observations in experimental animals. Taube and Essex [2] have reported in detail the pathological changes in dogs which received fatal doses of rattlesnake venom intravenously. The lesions ordinarily found in man follow subcutaneous administration and are not as intense or widespread as those found by Taube and Essex. The dogs used by these investigators died in from 8 minutes to 20 hours following the injection of venom, and showed petechial and massive hemorrhagic lesions which involved practically all the serous and mucous membranes of the body, as well as being distributed throughout many of the parenchymatous organs. Recovery is the general rule, as shown by a mortality rate of 8 per cent in a series of 50 cases of rattlesnake bite reported by Mitchell,[3] and 11.7 per cent in a series of 408 cases reported by Willson.[1]

Jackson's [4] observations on dogs which received subcutaneous injection of venom in the thigh are more nearly comparable with those on individuals bitten by rattlesnakes. Postmortem examination revealed a local, spreading hemorrhagic lesion in the tissues about the site of injection. The regional lymph nodes were in-

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tensely hyperemic and occasionally necrotic. The abdominal lymph nodes were involved later.

The pathological lesions produced by subcutaneous injections of rattlesnake venom into the monkey have not been described, as far as we know. The object of this paper is to report the gross and microscopic lesions in the *Macaca mulatta* monkey resulting from the subcutaneous administration of a lethal dose of rattlesnake venom.

Material and Methods

Nine young *Macaca mulatta* monkeys weighing from 2770 to 3950 gm. were given subcutaneous injections of *Crotalus* venom into the lumbar regions. The dry powdered venom was dissolved in physiological saline to make a 1 per cent solution. This toxic solution was divided into two equal parts and injected into both lumbar regions. The dose varied from 7 to 10 mg. per kilo of body weight.

The animals were autopsied on an average of 2 hours after death. Tissues were preserved in Zenker's fluid, formalin and 80 per cent alcohol. Sections were stained with Delafield's hematoxylin and eosin, Heidenhain's aniline blue stain, the scharlach R stain for fat, and by the phosphotungstic acid hematoxylin method.

Clinical Observations

Immediately after the injection of venom into the subcutaneous tissues the monkeys became disturbed and excited. After being released they ate, drank and behaved about as usual for several hours. A visible subcutaneous swelling and reddening of the skin about the site of injection gradually developed. After several hours the animals began to appear ill, as evidenced by weakness, torpidity and disinclination to eat. This condition increased in severity until finally death occurred at an average of 36.5 hours after injection. There was never any vomiting or passing of either bloody stools or bloody urine.

Autopsy Observations

The skin was discolored dark red in the lumbar region at the site of injection, and this appearance extended from this point into the anterior abdominal wall. When the skin was incised intense

hemorrhagic edema was found to be much more extensive than the discoloration of the overlying epidermis would indicate. This edematous process extended from the midthoracic dorsal region down to the buttocks, and occasionally as far as the knees. On the ventral surface of the body it involved the subcutaneous tissues from the groin up to as high as the middle of the thorax.

The subcutaneous connective and adipose tissues were four to six times their usual thickness. The cut surface was intensely red and thin bloody fluid ran out freely. The advancing edges of the lesion were edematous but the fluid was colorless or pale pink. The lesion was limited in depth by the deep fascia covering the abdominal, thoracic and lumbar muscles.

The regional lymph nodes were swollen and red. This was most marked in the inguinal and iliac nodes, but was quite noticeable in the axillary and preaortic nodes in most of the animals.

On opening the heart the most striking lesion was the presence of small patches of hemorrhage in the left ventricle beneath the endocardium, usually in the interventricular septum near the aortic ring. This was seen in 6 of the 9 animals. Usually slight in extent, in 1 animal it also involved the papillary muscles of the cusps of the mitral valve. In 1 animal also there were subendocardial hemorrhages in both auricles. In 8 of the monkeys the heart's blood was unclotted.

The lungs were well aerated, pink in color, and in 1 of the monkeys there were a few, small, subpleural hemorrhages.

The peritoneal cavity did not contain an excess of fluid. The visceral blood vessels were collapsed and contained very little blood. The intestines were a pale grayish color.

The liver was not unusual, except for a mild passive congestion in 3 animals. In 1 animal there were several small petechial hemorrhages along the anterior border.

The spleen was usually small, firm, and a slate gray in color. The cut surface was red and dry, and very little blood could be scraped from the surface.

The kidneys were pale but showed no other gross changes. In several animals the urinary bladder contained a small quantity of clear, straw colored urine.

The pancreas and adrenals appeared to be normal.

The gastro-intestinal tract showed no constant lesion. One

animal had a small, acute gastric ulcer measuring 0.5 cm. in diameter. Two others showed several small petechial hemorrhages in the submucosa of the colon.

The brain and upper part of the spinal cord were examined in each animal, and the only change of note was a slight congestion of the cerebral vessels.

Histological Observations

Skin and Subcutaneous Tissues at Site of Injection: There was a marked infiltration of the subcutaneous tissues with erythrocytes and considerable edema. The connective tissue fibers were fragmented and widely separated, and their nuclei stained poorly. Small blood vessels were seen in which the endothelial lining cells were swollen and their cytoplasm was eosinophilic and granular. The nuclei of these cells showed various retrogressive changes. Actual ruptures in the walls of these vessels were seen in places. Many vessels contained thrombi which partially or completely occluded the lumen. Accompanying the necrotic changes in the vessel wall were usually a few, scattered polymorphonuclear leukocytes. The capillaries were greatly distended and in some places appeared to be actually ruptured. Some showed homogeneous thrombi in their lumens. The extravasated erythrocytes were somewhat swollen and spherical, and stained very lightly with eosin. There were scattered collections of polymorphonuclears, lymphocytes and a few mononuclear cells, none of which contained phagocytosed erythrocytes.

The reaction was less severe at the edge of the lesion. There were fewer erythrocytes, but still a considerable quantity of faintly pink staining exudate was seen in the tissues. The fibrous connective tissue strands were not fragmented and nuclei of the fibrocytes stained normally. The capillaries and small blood vessels were greatly dilated but necrotic or thrombotic lesions were not seen.

The overlying epidermis showed no pathological changes except in 1 animal which lived 6 days and which developed a large area of necrosis at the site of injection.

Lymph Nodes: The nodes showing the most advanced and intense reaction were the inguinal, internal iliac and abdominal preaortic nodes. A similar but less intense reaction was found in

the axillary, and occasionally in the tracheobronchial and submaxillary nodes. In its most intense form there was great dilatation of the medullary sinuses which were filled with erythrocytes and large mononuclear phagocytic cells, some of which were distended with as many as fifteen to thirty red blood cells. The lesion was much less marked in the cortex of the nodes. The peripheral sinus, although usually slightly dilated, contained only relatively few monocytes and lymphocytes, and occasionally a few erythrocytes. The medullary blood vessels and lymphatics were dilated but no actual breaks in the vessel walls were found.

In some lymph nodes showing the most intense reaction there was a patchy infiltration with polymorphonuclear leukocytes. In places there was actual suppuration.

Lymph nodes in which the reaction was less severe showed mainly a proliferation of large monocytes in the lymph sinuses of the medulla. Some of these monocytes contained a few brown pigment granules and erythrocytes, but most of the cells contained very little phagocytosed material.

Heart: There was usually a moderate amount of myocardial fragmentation. In 6 animals there were small extravasations of blood into the subendocardial tissues of the left ventricular septum. These collections of erythrocytes elevated the endocardium and separated the superficial myocardial fibers. In 2 animals there was an infiltration with polymorphonuclear leukocytes. In 3 there was a well marked fatty degeneration of the myocardium; this was most marked in the superficial fibers close to the hemorrhagic lesion and was not seen in the more distant fibers.

Aorta: No pathological changes were found except in 1 animal where small deposits of fat were situated in the deeper part of the intima.

Lungs: The alveolar sacs were well distended with air and in 4 of the 9 animals this suggested the presence of a slight degree of emphysema. The alveolar capillaries were not unduly distended.

Liver: The venous sinusoids were slightly dilated with blood. In several animals the Kupffer cells seemed somewhat swollen, but this change was not prominent. The liver cells showed a moderate amount of granular degeneration. A small amount of fat was demonstrated in the liver cells of 4 animals and a large amount in 1.

Kidneys: Sections stained with Delafield's hematoxylin and eosin and with Heidenhain's aniline blue stain failed to reveal any pathological changes aside from slight granular degeneration of the tubular epithelium. The endothelial and epithelial cells of the glomerular tuft appeared normal, except for possibly some slight swelling, and were separated in the usual manner by a thin basement membrane. Sections stained with scharlach R showed a moderate increase of fat stored in the form of small globules in the collecting and convoluted tubules in 3 animals.

Gastro-Intestinal Tract: This was essentially normal throughout. In 2 animals small extravasations of blood were present in the submucosa of the colon. A small acute ulcer of the gastric mucosa was found in 1 animal.

Brain and Spinal Cord: There was slight congestion in the small blood vessels of the meninges, gray and white substance of the brain, and of the spinal cord. The cellular changes were not marked but there was evidence of some chromatolysis.

Discussion

It is evident from these observations that rattlesnake venom administered subcutaneously acts as a powerful tissue irritant. In its stronger concentrations it causes actual dissolution of blood vessels and connective tissue. At the advancing edge of the lesion, where the venom is somewhat diluted with tissue fluid, no necrotic vascular lesions are seen and the dilated capillaries and venules show only a somewhat swollen endothelial lining. The altered permeability of these vessels to fluids is evidenced by marked edema in the surrounding tissue. It is probably from this region that maximum absorption of venom-containing fluid takes place.

Drainage from the local lesion is partly by way of the regional inguinal lymph nodes. Here the diluted venom causes great dilatation of the medullary sinuses and proliferation of the reticuloendothelial cells. The venom is attenuated sufficiently so that necrotic or thrombotic lesions are not found in the blood vessels of these lymph nodes. However, the many free and phagocytosed red blood cells in the medullary lymph sinuses are evidence of the increased permeability of these vessels, which is indicated microscopically by only slight swelling of the vascular endothelium. From the regional lymph nodes the venom-containing lymph

passes along the iliac, preaortic and mediastinal chains of nodes.

Widespread lesions in the organs supplied by the systemic circulation were not striking. There was moderate granular degeneration of cardiac musculature and of the proximal convoluted and collecting tubules of the kidney. None of the exudative or hemorrhagic lesions of glomerular tufts, as described by Pierce [5] following the intravenous administration of venom into rabbits, were seen. Apart from a rather indefinite swelling of the glomerular endothelial cells, no constant lesion was present.

Mitchell and Reichert [6] first pointed out that snake venom is a complex material, both as to chemical and as to physiological properties. Flexner and Noguchi [7, 8] established the presence of agglutinins and hemolysins and presented evidence suggesting the presence of a neurotoxin and an anticomplementary body. The cytolysin which causes retrogressive changes in various cells, particularly those of the vascular endothelium, is undoubtedly the hemorrhagin referred to by Flexner and Noguchi. They claimed that the hemorrhages all take place by actual dissolution of the walls of vessels and never by diapedesis of erythrocytes. We have seen these tears in the smaller blood vessels and capillaries, but many vessels show only some degree of damage, as evidenced by nuclear changes and a swollen, indistinct vessel wall infiltrated with a few polymorphonuclear leukocytes. There is no actual rupture although there are erythrocytes in the vessel. It appears, therefore, that *Crotalus* venom may injure vascular endothelium to an extent, varying in proportion to its concentration, from merely a functional change which allows increased permeability to fluids and diapedesis of red blood cells, to actual necrosis, intravascular thrombosis and rupture of the wall.

Mitchell and Reichert [6] and Rogers [9] have described a profound fall in blood pressure after administration of crotalin, and Rogers noticed that this was accompanied by marked portal vascular dilatation. Taube and Essex [2] have redirected attention to this generalized vascular dilatation and point out that the gross and microscopic lesions following intravenous administration of venom are identical with the pathological lesions Moon [10] has suggested are present in the shock syndrome.

While the results obtained seem to point to the conclusion that shock is the major factor in the death of an animal injected with

crotalin intravenously, the evidence is not so clear for this assumption following subcutaneous administration. Moon [10] says: "The evidence does not support the idea that local loss of blood and/or fluid at the site of injury is an adequate explanation for shock." In our monkeys this local loss of blood and fluid was the most marked feature present. Any hemorrhage and edema appearing elsewhere was negligible in amount. The abdominal and thoracic viscera, instead of showing the marked engorgement, edema and hemorrhage associated with shock, were pale, their cut surfaces dry, and the blood vessels were collapsed. The local lesion, consisting of extensive subcutaneous hemorrhagic edema, may however, as Moon suggests, "contribute to the circulatory deficiency in proportion to the volume of blood and fluid lost." In our animals the subcutaneous hemorrhagic lesions were very extensive and involved about 50 per cent of the body surface.[11] It is possible that this local lesion may have caused enough disparity between the blood volume and the volume capacity of the vascular system to produce at least some degree of shock.

Before the primary cause of death can be ascribed to shock, however, it would seem essential to eliminate the possibility that some other fraction of the venom, such as the neurotoxin, may have a major or at least an important contributory part in fatal termination.

Summary and Conclusions

Lethal doses of rattlesnake (*Crotalus atrox*) venom were administered subcutaneously into the lumbar regions of 9 *Macaca mulatta* monkeys. Death occurred on an average of 36 hours after injection.

The outstanding lesion at autopsy was a marked hemorrhagic edema at the site of injection which spread extensively throughout the adjacent subcutaneous tissues. The regional lymph nodes were greatly swollen and hemorrhagic; the more distant nodes were less affected. In 6 of the 9 animals petechial hemorrhages into the subendocardial tissues of the left ventricle occurred. These were the only constant gross lesions. In 1 animal, small subpleural hemorrhages and subcapsular hemorrhages of the liver were found, and in 2 others there were similar lesions in the cecum.

Microscopically, in the region of the hemorrhagic edema necrotic

changes were found in the walls of the small blood vessels and capillaries. Many of these vessels contained ante mortem thrombi. The exudate in the surrounding tissues consisted mainly of erythrocytes and fluid but there were also scattered collections of polymorphonuclear leukocytes. The regional lymph nodes showed marked hyperplasia of the reticuloendothelial cells, which was most marked in the medullary portion. Here, erythrocytes lay free in the dilated sinuses and appeared to be phagocytosed by the reticuloendothelial cells. The hemorrhagic lesion in the left ventricle showed, in addition to the capillary dilatation and extravasation of blood into the tissues, fatty degeneration of the myocardium in the immediate region of the lesion. There was slight granular degeneration of the parenchymatous cells of the liver and kidney, and in 3 animals there was fatty degeneration of the kidney tubules.

Rattlesnake venom is a powerful tissue irritant which causes necrosis and actual dissolution of blood vessels with subsequent extravasation of erythrocytes and serum into the tissues.

Absorption seems to be mainly from the region of the advancing edge of the subcutaneous hemorrhagic lesion. At this point the venom-containing edematous fluid seems to be absorbed by the lymphatics and passes up the inguinal, iliac and mediastinal chains of nodes.

Shock seems the most probable major factor in the death of animals injected with venom intravenously, but the evidence is not so clear for this assumption following the subcutaneous administration. Our animals showed no generalized vascular lesions, only a subcutaneous loss of blood and fluid, so extensive however that it might be considered capable of at least producing some degree of shock.

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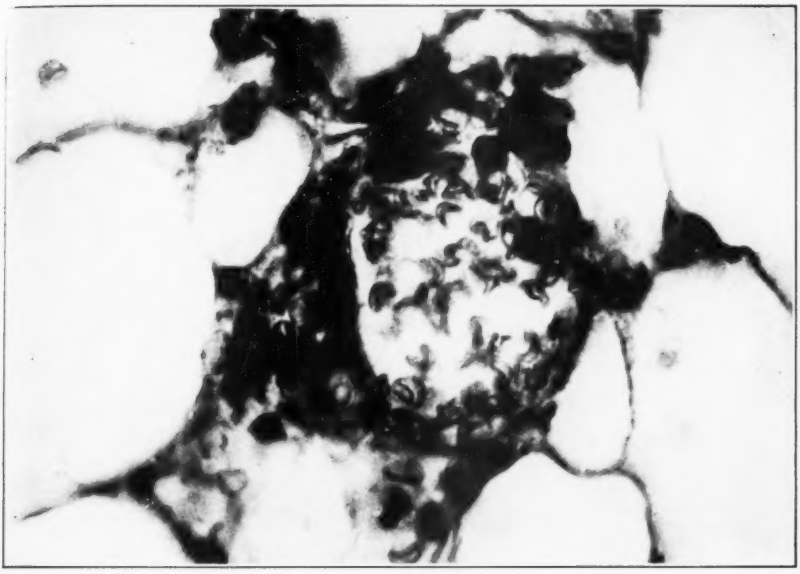
DESCRIPTION OF PLATES

PLATE 73

FIG. 1. Small blood vessel in the subcutaneous adipose tissue near the site of crotalin injection. The wall of the vessel is swollen and indistinct and erythrocytes are seen passing through by the process of diapedesis. × 850.

FIG. 2. Blood vessel in the region of subcutaneous hemorrhagic edema. An ante mortem thrombus is attached to the endothelium and partially occludes the lumen. The wall is ruptured in one place and erythrocytes are infiltrating the adjacent tissues. × 600.



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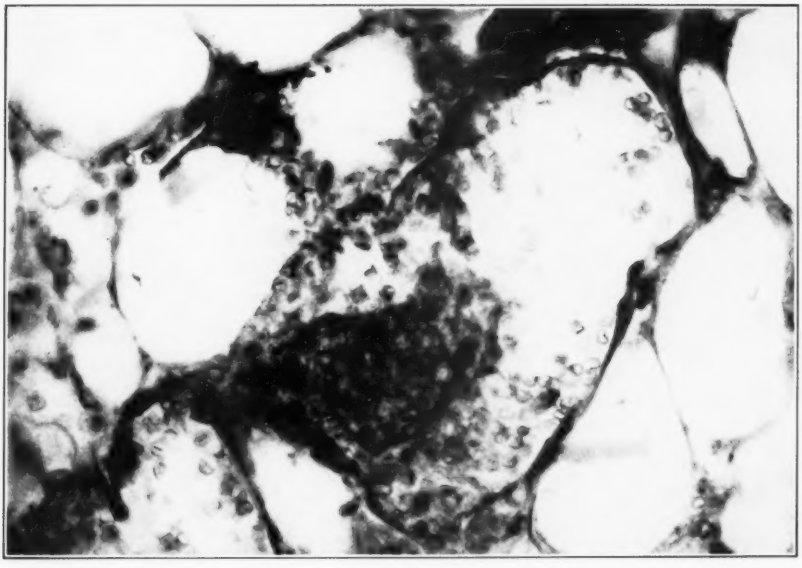


2

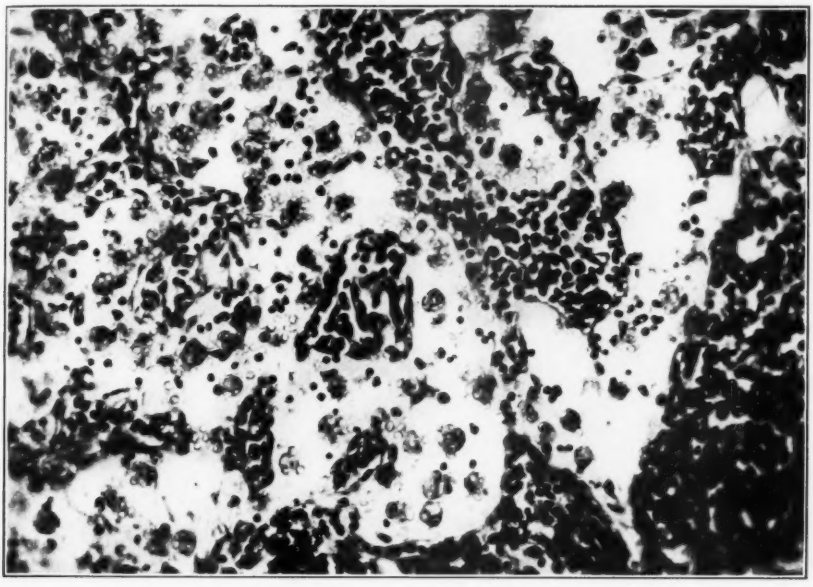
Fidler, Glasgow and Carmichael Injection of Rattlesnake (Crotalus) Venom

PLATE 74

FIG. 3. Medullary portion of an inguinal lymph node. The lymph sinuses are dilated and contain many erythrocytes, some of which are seen within large phagocytic mononuclear cells. × 400.

FIG. 4. Myocardium of the left ventricle in the region of the intraventricular septum. The endocardium is elevated by a collection of erythrocytes. Similar collections are seen separating the underlying bundles of myocardial fibers. × 400.

VAMPIRE



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Fidler, Glasgow and Carmichael Injection of Rattlesnake (Crotalus) Venom

MENINGOCOCCUS ENDOCARDITIS AND MYOCARDITIS *

Report of a Case with Unusual Lesions in the Arterial Tree

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The chief interest in the following report lies in the presence of peculiar lesions in the small arteries and arterioles which were found in a case of meningococcus endocarditis and myocarditis.

Meningococcus endocarditis is a rare complication of meningococcus meningitis. Herrick,[1] in reporting an outbreak of 208 cases of epidemic cerebrospinal meningitis in a camp, found complicating endocarditis only once (verified at autopsy). Smithburn and his co-workers,[2] in reporting the Indianapolis epidemic of meningococcus meningitis, reported clinical endocarditis as a complication in only 5.5 per cent of 144 cases. It is rather surprising that approximately only one-half of the cases of meningococcus endocarditis reported in the literature, and verified at autopsy, were associated with meningitis. Meningococcus myocarditis is even rarer, judging by the small number of cases reported. For this reason, the pathological lesions observed in a case of meningococcus endocarditis and myocarditis, which were not associated with meningitis, are of especial interest. The arterial lesions found on microscopic examination make the case unique.

Analysis of 20 autopsy records in the cases reported by Herrick,[1] Hyland,[3] Weindel,[4] MacMahon and Burkhardt,[5] Stevenson,[6] Willius and Eaton,[7] Melnotte and Fort,[8] Lemierre *et al.*,[9] and in the reviews and case reports of Rhoads,[10] and Gwyn,[11] reveals that in 11 cases the mitral valve alone was involved, and in 3 other cases the lesions of the mitral valve were accompanied by aortic vegetations. The predilection of the *Streptococcus viridans* and other types of bacteria for scarred valves is well known, but an analysis of the cases of meningococcus endocarditis available in the literature shows that a previous history of rheumatic fever was given in only 3 of the 20 cases reviewed, and in a 4th, sclerosis

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of the mitral and aortic valves was noted. However, this apparently relatively low incidence is apt to be misleading, as it was stated definitely in only 6 reports that there was no history of rheumatic fever. Where evidence of damage to the valves due to previous attacks of rheumatic fever was reported, vegetations were found on the valves affected by the rheumatic process at autopsy.

In a recent review of meningococcus myocarditis Saphir,[12] noted in 5 cases lesions suggestive of Aschoff nodules in the myocardium. In his own cases the typical lesions consisted of a hemorrhagic exudate, the early appearance of endothelial leukocytes, destruction of muscle fibers, and the presence of meningococci in the myocardium. Also, there were foci of necrosis similar to those seen in the myocardium in subacute bacterial endocarditis. Meningococcus myocarditis is usually associated with endocarditis, as in the case here presented.

Report of Case

Clinical History: Mrs. E. C., aged 40 years, a white Canadian housewife, was admitted to the hospital with a complaint of weakness in the legs, anorexia, vomiting of greenish fluid and abdominal cramps. The history, however, is somewhat unreliable as the patient had a depressive psychosis.

Three years before admission the patient had attacks of dizziness associated with a sense of rotation. During the 6 months previous to admission to the hospital she had had severe epistaxis at least once a week, associated with marked anorexia and abdominal cramp-like pains. Three months before admission she had severe pain in the vertebra prominens, associated with redness and swelling, and from this region the pain spread down the trunk and the legs. Weakness in the legs, swelling of the ankles, and vomiting of greenish colored fluid without relation to the abdominal cramps, commenced 7 weeks before admission. During the last week she had had a dry, non-productive cough and diarrhea. On the day of admission she had two chills.

At the age of 9 years the patient had rheumatic fever and chorea, and at 22 years, a recurrence of rheumatic fever.

Physical Examination: Emaciation was marked. There was no postnasal discharge and no evidence of meningeal irritation. Occasional crackling râles were heard at the base of both lungs. The heart was moderately enlarged and there were loud aortic and mitral systolic and diastolic murmurs. The blood pressure was 135/65 mm. Hg. The liver and spleen were moderately enlarged to palpation. A point of unusual interest was a fairly marked chill experienced by the patient when the spleen was palpated. There were no petechiae in the skin. Clubbing of the fingers was slight.

Laboratory Examination: The leukocyte count was 25,000, with a differential count showing 62 per cent neutrophils. The red cell count was 5,000,000. The hemoglobin was 50 per cent, and the blood smear examination indicated an advanced secondary anemia. The urine showed a trace of albumin and a few granular casts. A blood culture taken the day following admission was

negative. Electrocardiograms revealed only occasional auricular premature beats.

Provisional Diagnoses: (1) Chronic rheumatic myocarditis and endocarditis; (2) bilateral bronchopneumonia; and (3) subacute bacterial endocarditis.

Course of Illness: The patient steadily lost ground, running a high fever (100° F. to 105° F. (R)) with drenching sweats. A blood culture taken 1 week before death showed on the 3rd day of incubation a growth of Gram-negative diplococci. These organisms grew only on media containing either blood or serum. They fermented dextrose and maltose but not saccharose, and were agglutinated by polyvalent antimeningococcal serum. They were therefore identified as meningococci.

Approximately 2 weeks following admission, and 4 days before death, the patient developed a right-sided hemiparesis, upper motor neurone in type. This was considered to be due to a cerebral embolism. Lumbar puncture showed a clear spinal fluid under normal pressure which was negative for globulin and which contained 30 neutrophils per cmm. A lumbar puncture the following day was essentially similar. Culture of the spinal fluid was negative for meningococci. Prontylin, 80 grains by mouth daily, was administered but had no effect. During the final 3 days the patient was drowsy, unresponsive and incontinent of urine and feces. The signs of bronchopneumonia became more widespread, and after a short period, during which respirations were shallow and the blood pressure fell, the patient died.

Final Diagnoses: (1) Meningococcus endocarditis; (2) bronchopneumonia, bilateral; and (3) depressive psychosis.

Postmortem Examination

Autopsy (A–478/37) was performed approximately 4 hours postmortem. The body was markedly emaciated. Numerous pin-point petechiae were scattered over the right forearm and region of the elbow joint. The fingers were slightly clubbed. A small amount of pitting edema was present in both feet.

The heart (Fig. 1) was slightly enlarged and weighed 380 gm. There were several, scattered, pin-point petechiae in the pericardium. The right auricle and right ventricle were considerably distended with fluid blood and postmortem clot.

The mitral valve admitted the tip of the index finger, being markedly thickened by firm fibrous tissue, particularly along the free margins of the leaflets. The chordae tendineae were similarly thickened by fibrous tissue and were markedly shortened. The posterior papillary muscle inserted directly into the edge of the posterior mitral leaflet. Covering practically the whole of the auricular surface of this leaflet was a markedly irregular, moderately firm elastic mass of yellowish pink vegetations which was firmly adherent over an area measuring 5 by 5 cm. The larger

vegetations projected approximately 1 cm. from the surrounding endocardium and hung down into the left ventricle. Similar but less extensive vegetations were present on the opposite leaflet close to its free margin, and also on its ventricular surface, to a slight extent. The aortic cusps showed only slight thickening and fibrosis, particularly at their commissural extremities. The tricuspid and pulmonary valve leaflets were normal. All the valve orifices were of approximately normal measurement, including that of the mitral valve, which measured 8.5 cm. The degree of thickening and consequent rigidity of the mitral valve leaflets, however, were such that stenosis and insufficiency must have been present, apart from the effect of the vegetations. A direct smear of the vegetations showed myriads of Gram-negative cocci, the majority of which were in pairs. Cultures of the vegetations showed an organism identical with that recovered from the blood ante mortem.

There were a few insignificant arteriosclerotic changes in the coronary arteries which had not appreciably narrowed their lumens.

The myocardium was soft, flabby and slightly opaque, suggesting cloudy swelling. Numerous patches of fibrosis, measuring from 2 to 7 mm. in diameter, were present throughout the myocardium of the interventricular septum and left ventricle. These were most numerous toward the base of the heart. There were no other significant lesions in the heart.

There was a bilateral pleural effusion of approximately 500 cc. A moderately well advanced pneumonia was present in the hilar region of the left lung. The liver showed slight passive congestion, and there was a fairly recent infarct in the spleen. The remaining organs were free of significant lesions. There was no evidence of meningitis, and on section of the brain nothing on gross examination was found to account for the left-sided hemiparesis noted in the history. Cultures from the pleural fluid, the consolidated portion of the lung, the nasopharynx and the meninges were negative for meningococci.

Microscopic Examination

All sections were stained with hematoxylin and eosin. In addition, sections of the vegetations and myocardium were stained by

Glynn's modification of the Gram stain, Giemsa's method, and the azure B bromide stain. Sections of the myocardium were also stained with Weigert's elastic tissue, Mallory's phosphotungstic acid hematoxylin, and Masson's trichrome stains.

The vegetations consisted of irregular masses of platelets and reticular fibrin which showed varying degrees of organization. Several almost completely organized masses were covered by endothelium. Distributed throughout the vegetations were patchy aggregations of neutrophils with lesser numbers of lymphocytes. Among these infiltrations were small grayish masses which, with bacterial stains, proved to be myriads of intra- and extracellular biscuit shaped diplococci, typical of meningococci. Difficulty in staining the bacteria was experienced, and it was only with the azure B bromide stain that the presence of the diplococci could definitely be determined.

The mitral valve was greatly thickened throughout by dense fibrous tissue. Extending approximately two-thirds of the distance to the free edge of the leaflet from the base were small blood vessels, accompanied by macrophages and fibroblasts. The deeper portions of the vegetations in each instance showed evidence of organization. In the center of the thickest portion of the valve were granular grayish patches of necrosis.

Sections from the right and left ventricles and from the interventricular septum were essentially similar. The muscle bundles and individual muscle fibers were swollen and separated by slight edema. All the muscle fibers showed moderate cloudy swelling and, in patchy areas, fragmentation and loss of cross striations, particularly in the regions about the blood vessels. Moderate sized patches of old fibrosis were scattered throughout the myocardium. These had the appearance of healed scars of rheumatic myocarditis. A careful search for evidence of activity of the rheumatic process revealed none.

A moderate degree of diffuse neutrophilic and lymphocytic infiltration with perivascular concentrations was present. Lesser numbers of macrophages and eosinophils showed a similar distribution (Fig. 2).

Many of the smaller arteries, and particularly the arterioles, contained rounded, pink-staining granular masses which almost, but never completely, occluded their lumens. These masses, or

plugs, which did not stain for fibrin with the phosphotungstic acid hematoxylin stain, usually were covered by a single layer of swollen endothelial cells (Figs. 3 and 4). The appearance of these peculiar vascular lesions was by no means constant. It varied from lesions regularly rounded in contour and homogeneous, and about which there was only a slight or moderate inflammatory reaction, to those less clearly outlined, only partially covered by endothelium, and which were surrounded by a fairly marked inflammatory reaction consisting mainly of neutrophils and lymphocytes. The shadowy outlines of nuclei were frequently present in these masses. In one vessel there was an irregular swelling of the media with loss of nuclear staining. Through this area there was a rupture of the wall with hemorrhage into the perivascular tissues. Sections mordanted in phosphotungstic acid failed to show evidence of fibrin in any of the masses. The Weigert elastic tissue stain showed the elastic laminae to have disappeared from the blood vessel wall in the neighborhood of these masses. Bacterial stains on sections of the myocardium showed no bacteria.

Sections of the lungs, spleen, kidneys and the remaining organs confirmed the gross findings.

An interesting discovery was the presence of rounded, partially endothelialized intravascular plugs in many of the smaller arteries and arterioles of the pancreas, liver, adrenal and uterus. These were similar to those seen in the myocardium and were associated with a similar but less marked perivascular inflammatory reaction.

Sections of the brain confirmed the absence of meningitis and showed no lesion to explain the hemiparesis.

Anatomical Diagnoses: (1) Rheumatic sclerosis of the mitral and aortic valves, with stenosis and insufficiency of the mitral valve; (2) scarring of the myocardium (rheumatic); (3) dilatation and hypertrophy of the heart (right); (4) meningococcemia; (5) subacute meningococcus endocarditis; (6) acute myocarditis; (7) infarcts of spleen (old and recent); and (8) acute bronchopneumonia.

Comment

The most interesting lesions noted in the above case, and those which make it unique, are the lesions in the arterial tree.

Only 20 cases of meningococcus endocarditis, verified at au-

topsy, have been reported in the literature. The majority of these have been acute in their course. Mackarell,[13] reported 2 cases with illness lasting 12 and 16 weeks respectively. Whether the endocarditis had been present during the whole course of the illness is not clear. One of these individuals had had meningeal symptoms from the beginning, the endocarditis occurring, possibly, only as a terminal complication. Gwyn's case,[11] which had a clinical course lasting 8 months, presented most of the classical clinical signs of subacute bacterial endocarditis. Meningitis developed as a terminal event. There were vegetations characteristic of subacute bacterial endocarditis on the mitral valve. Sections of the myocardium showed the presence of an acute inflammation, characterized by a few neutrophils between the muscle bundles and edema of the interstitial tissue. Old perivascular sclerosis in the myocardium was suggestive of a rheumatic origin, although no Aschoff bodies were seen. An acute diffuse glomerulonephritis was also found. Gwyn, in labelling this a subacute type, is the first to have described this form of the disease. A comparison between the salient points of Gwyn's case and those presented in this paper shows a striking similarity, both between the fairly typical clinical histories in each, and in the site and character of the lesions in the respective hearts — each showing evidence of old rheumatic fever with localization of meningococci on the scarred valves.

Lemierre *et al.*,[9] reported a case in which the vegetations were located on the aortic valves. There had been no known antecedent rheumatic fever. Myocarditis, characterized by a proliferation of fibrous tissue, was also present.

The lack of associated meningitis in our case, and in approximately one-half of the cases reported, is worthy of note. This fact, and the increasing number of cases of meningococcus septicemia reported which fail to show evidence of a primary infection in the meninges, indicate a versatility of the organism beyond that signified by its name.

There is nothing in the clinical history or in the lesions observed at autopsy in this case to indicate clearly the portal of infection. Although cultures of the nasopharynx during life and at autopsy were negative for meningococci, it is not unlikely, especially in view of the postnasal discharge, that the meningococci were harbored there. As is well known, the infection of the heart valves in

classical subacute bacterial endocarditis may follow the liberation of organisms into the blood stream, with the *Streptococcus viridans* as the predominating organism. The existence of valves previously damaged by a rheumatic process, as in this case, appears to make this localization more probable.

Meningococcus myocarditis, although extremely rare as a single disease entity, is commonly present in association with meningococcus endocarditis. In this case the degree of cardiac embarrassment cannot be assessed accurately by the history or by the physical examination. The myocarditis, as judged by microscopic examination, certainly was of sufficient degree to have influenced unfavorably the course of the disease. However, the degree of involvement appears to be considerably less than that in the cases reported by Saphir.[12]

As regards the possible relationship of the myocarditis and the vascular lesions associated with it to the previous rheumatic fever, there was nothing to suggest activity of the rheumatic process. This is especially significant when an attempt is made to explain the peculiar vascular lesions. Gross, Kugel and Epstein,[14] in describing lesions of the coronary arteries and their branches in rheumatic fever, describe, among others, two forms of vascular lesion which might be compared to the peculiar vascular masses in this case. They describe "*arteriitis verrucosa*" and "*granular plugged vessels*." *Arteriitis verrucosa* occupied an intermediate position between a necrotizing arteriitis and intravascular thrombosis. The process, they state, would appear to have been a fibrinoid necrosis of the intimal layers with fusion of the various histological elements. This may lead to the deposition of platelet thrombi, producing the verrucous lesion in the arterial tree.

Granular plugged vessels, they suggest, are apparently produced by a proliferation of the vascular endothelium with swelling and granular transformation of the protoplasm, fusion of the cells and degeneration of the nuclei. Each of these two vascular changes described by Gross and his colleagues tally well, in several respects, with the appearance of those in our case. Neither, however, was associated with an acute inflammatory reaction and in neither is there evidence that the change was primarily medial, as it would appear to have been in at least some of the vessels in the present case.

A definite decision as to the origin of these lesions cannot be made with the evidence available. Three possibilities — embolism, thrombosis and medial necrosis — can be considered. Embolism is suggested by the acute inflammatory reaction about the masses and by the confinement of the lesions to the arterial side of the circulation. The subsequent shrinkage of the embolic masses and their partial endothelialization subsequently, would explain how embolism could have resulted in the picture as seen now. In considering thrombosis as a possibility, it is more difficult to conceive of the mechanism by which it would result in an appearance such as these lesions present. Further, none of the vessels was completely occluded. Medial necrosis and swelling as the result of the action of bacterial toxins on the muscle coats would appear to be a plausible explanation. Certainly, judging by the appearance of at least one medium sized artery in the myocardium, this appears to be what had taken place. If this be the process, why this change should have occurred in localized patches and produce fairly uniform rounded masses cannot readily be explained.

Summary

1. A case of subacute meningococcus endocarditis and myocarditis with postmortem examination is presented. Unusual rounded masses projecting into the lumens of small arteries and arterioles were found in the myocardium, and to a lesser extent in bacterial toxins.

2. The nature of these vascular lesions is discussed. Although no definite conclusion is reached, it is suggested that they may be the result of foci of medial necrosis secondary to the action of bacterial toxins.

3. Only 20 cases of meningococcus endocarditis with autopsy report have been reported previously. This is the 3rd case reported as subacute in nature.

Note: I am deeply indebted to Dr. G. Lyman Duff and to Dr. Philip Greey for helpful advice and assistance given in the preparation of this report. Dr. W. L. Holman and Dr. William Boyd made valuable criticisms. Dr. E. A. Linell examined sections of the brain. Dr. Duncan Graham permitted the use of the clinical history. Miss C. M. Westlake rendered invaluable technical assistance.

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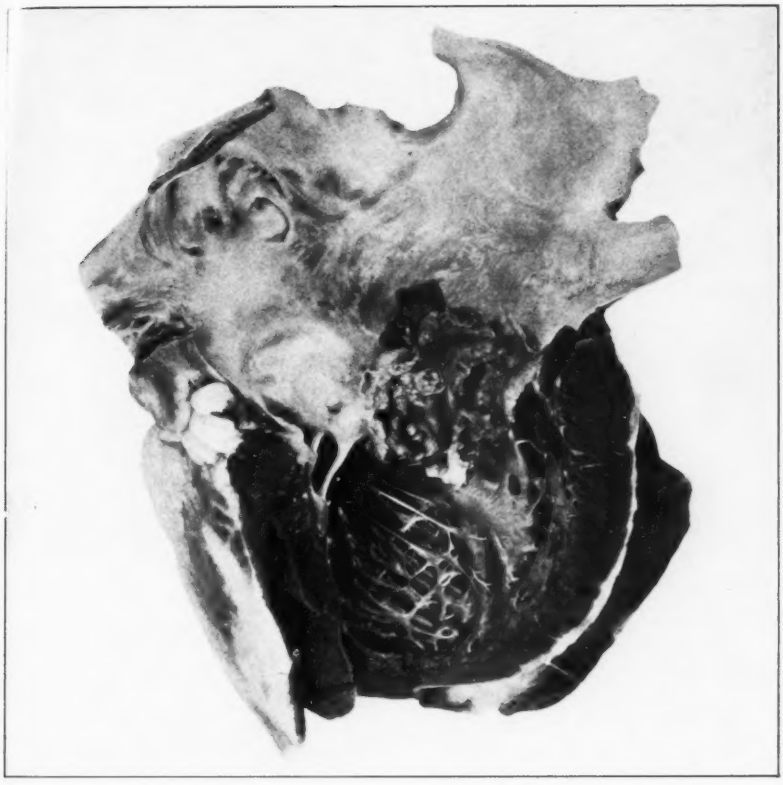
DESCRIPTION OF PLATES

PLATE 75

FIG. 1. Heart. View of the left auricle and ventricle with large, fairly tough vegetations on the posterior mitral leaflets. The evidence of marked damage to the valve by previous rheumatic fever is shown.

FIG. 2. Myocardium. A typical area of fibrosis with inflammatory reaction. A mild neutrophilic and lymphocytic infiltration is present in the perivascular space. Lymphocytes and macrophages are distributed throughout the area of fibrosis and among the muscle fibers. × 100.



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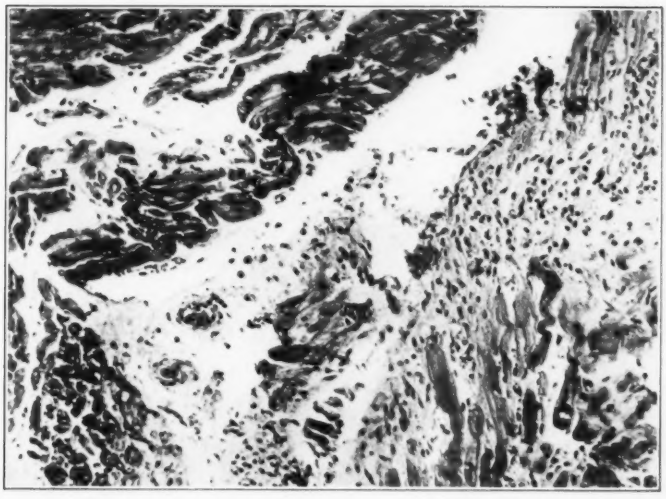

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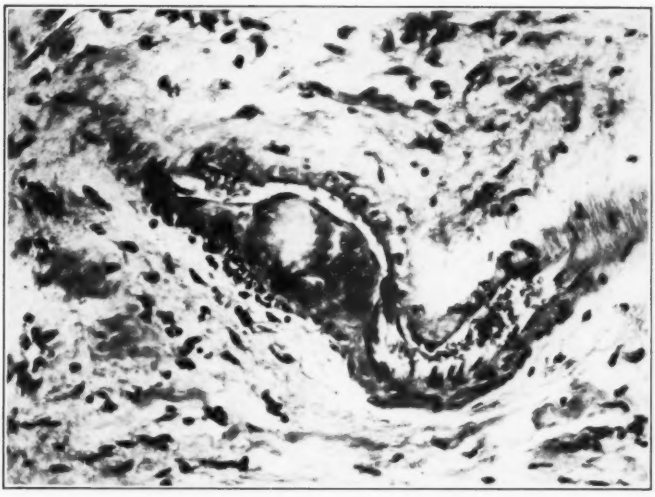
PLATE 76

FIG. 3. Myocardium. A plugged vessel in longitudinal section. The mass in the vessel is covered by endothelium. There is a perivascular infiltration of neutrophils, eosinophils and plasma cells. Fibrosis and a macrophage reaction are present in the outlying portions of the field. × 200.

FIG. 4. Myocardium. A plugged vessel in transverse section. The mass which almost completely fills the lumen is partially covered by swollen endothelial cells. The surrounding inflammatory cells are neutrophils and lymphocytes, with a few macrophages. × 450.



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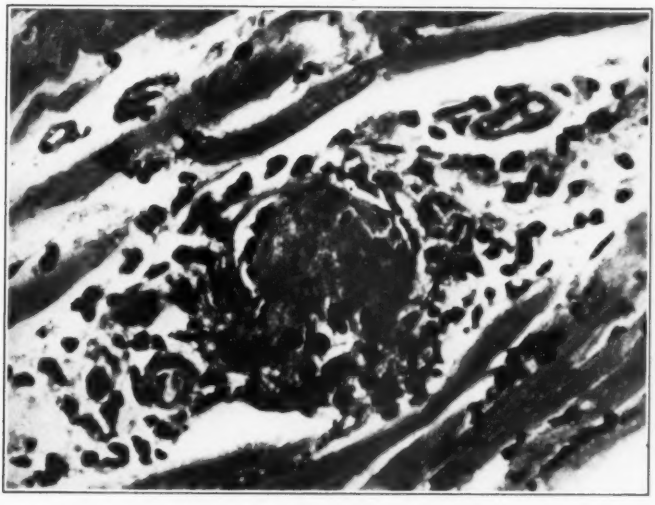

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Whillans Meningococcus Endocarditis and Myocarditis